STUDIES ON ALCOHOL DEHYDROGENASE NULL ALLELES FROM NATURAL POPULATIONS OF *DROSOPHILA MELANOGASTER*

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Declaration

The research described in this thesis, except where acknowledged, is the original work of the author.

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Abstract

Alcohol dehydrogenase (*Adh*) null activity alleles were detected in seven Australian natural populations of *Drosophila melanogaster* at frequencies up to 3.9%, but with an average of 1.3% in 1983. These values compare with a previously reported frequency of 0.09% for *Adh* in a North Carolina (U.S.A.) and a London (U.K.) population. Null alleles persisted in most of the populations over the next three years. Five of the Australian populations were located in Tasmania and nine *Adh* null alleles were isolated from three of these populations in 1983, seven from three in 1984, and seven from one population in 1985. All the 1983 and 1984 null allele bearing chromosomes were found to be viable with *Df(2L)64j*. Of the 23 isolated, 19 were homozygous viable and all were found to lack ADH activity and survive exposure to pentenol vapour. Three of the nine null bearing chromosomes isolated in 1983 were homozygous lethal and two of these were lethal in combination. The remaining six 1983 *Adh* null alleles failed to show interallelic complementation with each other or with the EMS-induced null, Adh^{n11}. All of the homozygous viable null alleles were unable to form active heterodimers with either Adh^F or Adh^S alleles, while of 12 tested by ELISA, none were found to produce any detectable ADH cross-reacting material (CRM). In addition, Western blotting confirmed the lack of ADH CRM in the six homozygous viable null alleles isolated in 1983. Heterozygotes between the null alleles and standard Adh^F and Adh^S alleles had intermediate ADH activity and CRM levels. The null homozygotes had similar, but slightly lower, mortalities on ethanol supplemented media to an artificially-induced null allele, Adh^{n10}. The Tasmanian and Adh^{n10} mortalities were considerably greater than the Adh^F or Adh^S homozygotes tested. Southern blot analyses of an 11.8 kb region

surrounding and containing the *Adh* gene failed to detect any insertions or deletions in the *Adh* null alleles as compared to an active Adh^S allele isolated from one of the Tasmanian populations in 1983. The mRNAs produced by the *Adh* null alleles were found to differ from wild-type by being longer (100 bases) and present in reduced amounts (10%). It is suggested that the mutation(s) responsible for the loss of ADH activity in the Tasmanian *Adh* null alleles occurs at one of the intron-exon splice site junctions of the *Adh* gene, thereby affecting the processing and translation of the null *Adh* transcript. No heterogeneity was detected in the biochemical and molecular properties of the separately extracted Tasmanian *Adh* null alleles and this suggests a similar type of mutation in each. The origin and maintenance of the *Adh* null alleles in the Australian populations is discussed in the light of this evidence.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Literature Survey: Null Alleles in Natural Populations

The application of electrophoretic techniques to population studies, pioneered by Harris (1966), Johnson, Kanapi, Richardson, Wheeler, and Stone (1966), and Lewontin and Hubby, (1966) has allowed the investigation of the genetic structure of populations, and demonstrated the existence of considerable genetic heterogeneity in animals and plants. These studies have usually assumed that individuals exhibiting a single band of activity after electrophoresis and staining represented homozygotes with two copies of the same allele. Null activity alleles have been assumed to be sufficiently rare to be disregarded in studies of allelic variation at allozyme loci.

The occurrence of a null allele at an autosomal locus is likely to have a severe effect on the level of gene expression in diploids. For an enzyme locus, a reduction in enzyme activity of about 50% is expected in heterozygotes while in null homozygotes enzyme activity would be totally eliminated. From a selectionist viewpoint, such mutations should be rare with selection against null alleles balancing recurring mutation to null alleles.

This thesis describes the discovery and subsequent characterisation of alcohol dehydrogenase (*Adh*) null activity alleles found at, or close to, polymorphic frequencies in Australian populations of *Drosophila melanogaster*. The relatively high frequency of *Adh* null alleles was unexpected given the assumptions generally made about the frequency of null alleles in natural populations. Therefore, it is desirable to review the evidence for the occurrence and frequency of null alleles at enzyme loci in natural populations of eukaryotes.

This review forms the first part of this Chapter; in the second part I describe the *Adh* polymorphism in *D. melanogaster*. Together these

two sections are intended to provide a general background against which the findings of this study can be viewed.

Mohrenweiser (1981) has been unable to trace the origin of the term 'null', but it is used liberally and with some confusion. I define null alleles as allelic variants at structural enzyme loci which either encode for a nonfunctional protein or fail to produce a protein. The latter are the classical nulls, but as pointed out by Neel (1983) the distinction between the two classes is often dependent on the sensitivity of the immunological technique used. Therefore a more appropriate term is 'null activity allele' which can include all the variants lacking enzyme activity regardless of the nature of the mutation responsible. For the sake of simplicity I will use the term 'null allele' as a synonym for the longer 'null activity allele'.

Rick and Fobes (1975) conclude that a survey of the literature gives the impression that nulls tend to be infrequent in natural populations while Ferguson (1980) contends that null alleles are probably only found in polyploid organisms or at loci which have been duplicated. Nevertheless, the presence of null alleles has often been postulated to explain the homozygote excesses found in natural populations. Homozygote excesses, or a deficiency of heterozygotes, are detected when the observed homozygote and heterozygote genotype classes are compared to those at Hardy-Weinberg equilibrium. There are five conceivable hypotheses which, singly or in combination can account for such deviations: (1) population subdivision and Wahlund effects, (2) positive assortative mating of genotypes, (3) inbreeding, (4) selection against heterozygotes, and (5) undetected null alleles (Ayala, Valentine, Barr, and Zumwalt, 1974; Spiess, 1977). In the case of the undetected null allele or alleles, null/active allele heterozygotes are

mistakenly classified as active/active allele homozygotes thereby increasing the homozygote genotype frequency.

Heterozygote deficiencies have been reported in a variety of mammal and fish species (Manwell and Baker, 1969), but appear to be common in marine organisms, particularly marine molluscs. Zouros and Foltz (1984, as cited by Mallet, Zouros, Gartner-Kepkay, Freeman, and Dickie, 1985) have listed 27 species of marine bivalves from studies by various authors in which deficiencies have been observed. On the basis of this survey and their own studies, Mallett *et al.* (1985) maintain that heterozygote deficiency in marine bivalves varies with age and the loci sampled; some loci, e.g., leucine aminopeptidase are most likely to show significant deviations while others, e.g., glutamate oxaloacetate transaminase do not.

Milkman and Beaty (1970) were among the first to find a heterozygote deficiency in a marine mollusc population. They conducted a large-scale survey of *Mytilus edulis* mussel populations (4000 individuals) and detected a substantial deficiency at the leucine aminopeptidase (*Lap*) locus which they attributed to the presence of a null or silent allele. In two other studies of the same organism Koehn and Mitton (1972) and Boyer (1974) found individuals lacking LAP activity in populations with heterozygote deficiencies. Boyer (1974) had detected three individuals out of a total 4871 scored which showed no detectable LAP phenotype after electrophoresis and staining. Calculations based on the observed deficiency estimated that null allele frequencies would have to be in the range 0.07 to 0.16, assuming the null allele was the sole cause for the observed deviation. Similarly, Koehn and Mitton (1972) had observed three individuals out of 3000 with an apparent LAP null homozygote phenotype, although they concluded that

the derived null allele frequency of 0.001 would be too low to produce the observed magnitude of homozygote excess. Gartner-Kepkay, Dickie, Freeman, and Zouros (1980) also concluded that the departures from Hardy-Weinberg equilibrium that they observed in populations of *M. edulis* were too large to be explained by null alleles.

In contrast, Skibinski, Beardmore, and Cross (1983), in explaining heterozyote deficits at several *M. edulis* loci, found that a null allele hypothesis was better corroborated than the generally favoured explanation involving differentiation in a subdivided population. From a comprehensive survey of *M. edulis* populations in the British Isles these authors estimated that null allele frequencies would have to range from 0.003 to 0.083 to account for the observed departures from equilibrium. Using the equation $x = \sqrt{u/s}$ for mutation-selection balance, where x is the null allele frequency and with selection against the null homozygote, they calculated mutation rate (u) values of between 10^{-5} to 10^{-2}, given a selection coefficient (s) of one. These mutation rate estimates compared favourably with the reported estimates of allozyme mutation rates of *D. melanogaster* (Mukai, 1970; Tobari and Kojima, 1972). Overall Skibinski *et al.* (1983) concluded that the postulated null allele frequencies were consistent with mutation-selection balance, although given the predicted frequencies of nearly 10% at some loci they suggested a purely selective interpretation may be more appropriate. In addition, some individuals who lacked detectable enzyme activity were observed by Skibinski *et al.* (1983) and for two loci, esterase-D and phosphoglucose isomerase, some two banded phenotypes expected of null/active allele heterozygotes were found. However, in the absence of breeding data, the difficulty with evidence of this kind is being able to distinguish between physiological

inactivation, experimental degradation, and the presence of true null alleles.

To confirm the existence of a null allele, breeding studies are required in which phenotype segregation can be compared to that expected under a null allele hypothesis. Unfortunately, many marine organisms do not lend themselves to breeding experiments, although there have been a few successful studies. Mallett *et al.* (1985) conducted a series of pair matings of adult *M. edulis* mussels to determine the cause of heterozygote deficiencies in this species. They found one mating in which the segregation pattern of LAP allozymes in the F1 progeny was consistent with the presence of a null allele in one of the parents. In a series of elegant studies Siegismund (1985a,b) and Borowsky, Borowsky, Milani, and Greenberg (1985) have bred species of the amphipod genus *Gammarus* to test for the presence of null alleles in populations showing heterozygote deficits. Wild-caught egg carrying female *G. oceanicus* amphipods were maintained separately until the juveniles had hatched, after which the female and her F1 progeny were typed for electrophoretic variants at the mannosephosphate isomerase (*Mpi*) and phosphoglucose isomerase (*Pgi*) loci. From the observed segregation patterns both systems were determined to be encoded by single autosomal loci, each with five co-dominant alleles. In addition, a number of anomalous patterns were found for some broods in which the progeny were found to segregate with phenotypes incompatible with the apparent homozygous phenotype of the mother. These cases were explainable by the segregation of a recessive null allele at the *Pgi* and *Mpi* loci.

Borowsky *et al.* (1985) had found a consistent pattern of heterozygote deficiency for an amylase locus in a population of the salt marsh amphipod *G. palustris*. To determine if null alleles were

contributing to the deficiency they mated field-collected animals, electrophoresing both parents and progeny to test for anomalous segregation patterns indicative of a null allele. No anomalous broods were detected, although a number of potential null homozygotes had been identified in the original electrophoretic survey.

Other organisms showing heterozygote deficits at enzyme loci in which a null allele may be a contributing factor include the intertidal phoronid *Phoronopsis viridis* (Ayala *et al.*, 1974), the chiton *Sypharochiton pelliserpentis* (Freeth and Sin, 1986) and the eelpout *Zoarces viviparus* (Christiansen, Frydenberg, Gyldenholm, and Simonsen, 1974; Christiansen, Frydenberg, Hjorth, and Simonsen, 1976.)

It is interesting to note that assumptions concerning the rarity of null alleles has led to some circularity in arguments against their involvement in heterozygote deficiencies. For example, Boyer (1974) doubts if a null allele could be an adequate explanation because of the onus of justifying the required frequency of about 12% for null alleles while Tracey, Bellet, and Gravem (1975) conclude that null alleles are not encountered frequently enough to explain the deficiencies they observed at three separate loci. Often a null allele hypothesis is dismissed because no apparent null homozygotes are detected (Beaumont, Day, and Gade, 1980; Smith, Franics, and Jamieson, 1981) even though such homozygotes may be inviable or alternatively under strong selection.

Rick and Fobes (1975) concluded that null alleles are infrequent in natural populations on the basis of four surveys of polymorphic loci in the American eel, the horseshoe crab, and two species of *Drosophila* (Williams, Koehn, and Mitton, 1973; Selander, Yang, Lewontin, and Johnson, 1970; Johnson, Kojima, and Wheeler, 1969; Prakash, Lewontin, and Hubby, 1969).

In fact, as noted by Manwell and Baker (1970), what is surprising is the number of null alleles which have turned up in studies surveying relatively small numbers of animal and plant species. In humans there are currently over 100 metabolic diseases involving a specific enzyme defect which are due to null or low activity mutations (Raivio and Seegmiller, 1972; Mohrenweiser, 1981). Almost all of these defects are detectable in heterozygotes through a significant reduction in enzyme activity (usually approaching 50% of the expected value) and many can have severe effects on the health of the carrier (Neel, 1978; Mohrenweiser, 1981). Examples of null mutations of enzyme loci in man include the enzymes glyoxalase, esterase, and glutathione S-transferase nulls (Sparkes, Sparkes, Crist, and Anderson, 1983; Dissing and Eriksen, 1984; Laisney, Nguyen Van Cong, Gross, and Frezal, 1984).

In reviewing the literature to assess the extent of null allele occurrences I have discovered 36 publications dealing with studies of natural populations of 34 plant and animal species in which null alleles have been detected. Table 1.1 summarises the 36 studies (from Tables 1.2 to 1.6) in terms of the enzyme systems investigated. Overall, 36 enzymes were examined and out of a total of 152 separate investigations, 72 (47%) found evidence for null alleles in 24 different enzyme systems. It is worth noting that in many population studies detection of a null allele relies primarily on the occurrence of the phenotype expected of a null homozygote, as Hardy-Weinberg equilibrium calculations are often not made and segregation experiments are rarely carried out. The probability of not detecting a null homozygote with an allele frequency q and a sample size n is $(1-q^2)^n$ (Allendorf, Stahl, and Ryman, 1984), which remains high even when the null is present at polymorphic frequencies and a large number of individuals are surveyed

(e.g., n = 100, q = 0.05 or 0.01, then prob. = 0.78 or 0.99 respectively).

In addition, many workers will exclude such phenotypes from their data because of the uncertainty regarding the phenotype's origin (i.e., does it represent physiological inactivation or a true null allele?) or lump them together with other rare mobility variants and then calculate a single frequency. Guries and Ledig (1978) have noted that the reports of null alleles in the literature occur almost exclusively in systems using synthetic substrates which are not likely to occur *in vivo*, and argue that such null designations may therefore prove erroneous when electrophoretic conditions or staining procedures are modified.

Tables 1.2, 1.3, and 1.4 list studies on invertebrate and vertebrate, plant, and insect populations with evidence for a null allele. The estimates of null allele frequency represent the range of frequencies which were calculated and they do not imply that null alleles occurred at a locus (loci) in every population. These frequency estimates were calculated from the population data in a number of ways. Several studies (e.g., *M. edulis* populations) estimated the null frequency directly from an observed heterozygote deficiency while some (e.g., Selander and Yang, 1969) used a maximum likelihood method utilising information on the frequency of null homozygotes and the degree of heterozygote deficiency not involving active alleles. Others simply used the square root of the frequency of presumed null homozygotes.

One of the striking patterns of null allele occurrence is the large number of esterase nulls present both in plant and animal species. Of the 152 enzyme investigations summarised in Table 1.1, 23 (15%) report esterase nulls, while 45% (22/49) of all the nulls reviewed in Tables

1.2 to 1.4 occurred at esterase loci. This has been a phenomenon noted by many workers (Rick and Fobes, 1975; de Stordeur, 1976; Allendorf *et al.* 1984) and although it may reflect in part the greater number of investigations concerning esterase allozymes (see Table 1.1) the high variability of esterases across taxonomic lines is well known (Powell, 1975).

Gillespie and Kojima (1968) were the first to offer an explanation for the differential variability observed among enzyme systems such as esterases. They classified enzymes according to whether they were or were not involved in glucose metabolism. Glucose-metabolising (Group I) or more generally, specific substrate enzymes are reputed to be less variable than non-glucose-metabolising (Group II) or variable substrate enzymes such as esterases or phosphatases (Gillespie and Kojima, 1968; Kojima, Gillespie, and Tobari, 1970; Powell, 1975). Singh (1976) states (without quoting any data) that null alleles are far more commonly observed for Group II loci than for Group I. Table 1.7 lists the null alleles reported in this review in terms of the Group I and Group II designations of Kojima *et al.* (1970). Overall, accepting the few data and the heterogeneity present within each group, there appears to be a higher number of null reports per total enzyme investigations for Group II enzymes.

Johnson (1974) has extended the Gillespie and Kojima (1968) proposal by further dividing enzymes into three classes: variable-substrate, regulatory, and non-regulatory enzymes. Data from a wide range of vertebrate and invertebrate species indicate that the most polymorphic group of enzymes is the variable-substrate enzymes followed by regulatory enzymes with the non-regulatory enzymes being the least variable (Johnson, 1974; Powell, 1975). Analysing the data from this

review in a similar fashion demonstrates a pattern of null allele occurrence consistent with their finding, although considerable heterogeneity exists within each group (Table 1.8).

An impression gained from the studies reviewed is that null alleles tend to be associated with multilocus systems. Of the 26 reports of null alleles which provide information on loci number, 24 (92%) have two or more loci encoding for the same enzyme. The high frequency of nulls observed in the plant and animal studies reviewed may simply be a consequence of the functional redundancy associated with multilocus enzyme systems. There is good evidence supporting this view from studies of gene duplication in a variety of organisms. In many of these organisms null alleles are found at, or near to, fixation in populations as a result of the selective shelter provided by two or more loci; mutations can accumulate at one locus while the other(s) retains normal function (Wilson, Barber, and Walters, 1983).

Allendorf *et al.* (1984) describe a polymorphism for lactate dehydrogenase from the skeletal muscle of the brown trout (*Salmo trutta*) which is due to the presence of a null allele at the *Ldh-1* locus. Five LDH loci have been described in the trout, but only *Ldh-1* and *Ldh-2* are expressed in the skeletal muscle; they are paralogous genes that appear to have been duplicated in the ancestral salmonid. A population survey of 14 populations of brown trout in Sweden revealed *Ldh-1* null allele frequencies ranging from 0.11 to 1.0 as calculated from the null homozygote frequency. Null alleles are common at many duplicated loci in fish species (May, Stoneking, and Wright, 1979; Klar and Stalnaker, 1979; Stoneking, Wagner, and Hilderbrand, 1981).

Roberts and Baker (1973) believe that four tightly linked alpha-esterase loci of *Drosophila montana* (Table 1.4) are the products of gene duplication. One of the interesting things about this multilocus set is the linkage disequilibrium which exists among null and active alleles at these loci. Of the sixteen possible chromosome types containing an active or a null allele at each of the four loci, four particular types were found in natural populations with a frequency of 1.5 to 4 times higher than expected from independent assortment (Baker and Kaeding, 1981). This linkage disequilibrium has been observed in a number of geographically separate populations and is known to have been maintained in one population for five years (Roberts and Baker, 1973; Baker, 1975; Baker and Kaeding, 1981). Although the selective basis for this pattern has been recently challenged (Allendorf, 1983), it appears that there is an optimum number of active loci per chromosome with about 75% of chromosomes having two active alleles (Roberts and Baker, 1973; Baker, 1975).

Multilocus or duplicated gene systems are best viewed as a special case in considering the frequency of null alleles, as such systems are likely to tolerate null alleles at one of several loci encoding the same, or similar enzymes. In addition, by surveying the literature for reports of null alleles in natural populations I have found the cases in which null alleles occur frequently enough to be detected; these are likely to be the exceptions rather than the norm.

To accurately gauge the true frequency of null alleles in a natural population it is necessary to survey a large number of structural gene loci in a single organism. Voelker, Langley, Leigh Brown, Ohnishi, Dickson, Montgomery, and Smith (1980a) and Langley, Voelker, Leigh Brown, Ohnishi, Dickson, and Montgomery (1981) were the first to

systematically screen natural populations of an organism (*D. melanogaster*) for the presence of null alleles at allozyme loci. Soon after, Allendorf, Knudsen, and Blake (1982) screened populations of ponderosa and red pine with a similar aim. The enzyme systems examined by Voelker *et al.* (1980a) were generally (16 out of 25) controlled by a single locus, although this was not the case for Allendorf *et al.* (1982) where only 10 enzymes were listed as having a single locus.

Voelker *et al.* (1980a) and Langley *et al.* (1981) used a tester stock method to survey 25 enzyme loci for null alleles. This procedure did not rely on the presence of a null homozygote or a heterozygote deficiency to indicate the existence of a null allele. Instead, a chromosome extracted from a wild-caught fly was made heterozygous with each of two mobility variants at the locus in question. Failure of the extracted allele to produce a heterozygous phenotype when heterozygous with either mobility variant suggested a null allele. Specifically, Voelker, Langley and co-workers (1980a, 1981) isolated second and third chromosomes by singly crossing wild-caught males to females of a balanced lethal stock (*Sm1, Cy/bw*^{v1}*; TM6, Ubx/Sb*). An F1 male from this cross (*Cy*/+; *Ubx*/+) was then backcrossed to females of the balanced lethal stock. Male progeny (*bw*^{v1}/+; Ubx or *Sb*/+) from this second cross were then mated to females of the tester stocks; a tester stock consisted of a dominantly marked balancer chromosome and a tester chromosome with electrophoretic mobility alleles different from the balancer at selected loci. Progeny marked either with the balancer phenotype or wild-type were classified after electrophoresis. Those alleles not forming a normal heterozygous phenotype with the tester alleles were designated putative nulls. Null alleles occurring on the X chromosome were tested for by directly crossing wild-caught males to females of the appropriate tester stock.

Table 1.5 lists the null allele frequency data of Voelker *et al.* (1980a) and Langley *et al.* (1981) for a *D. melanogaster* population located in England (London) and one in North Carolina (Raleigh). Twenty-five enzyme loci were screened, five of which were X-linked. No nulls were recovered at the five X-linked loci in either population, while the mean frequency of nulls at the 20 autosomal loci was 0.0025 and 0.0023 for the Raleigh and London populations respectively. The lack of nulls on the X-chromosome was statistically significant, even when heterogeneity among the autosomal loci was taken into account, and this was attributed to hemizygous effects. Overall, the distribution of null alleles in both populations was very similar and the authors concluded that the frequencies were consistent with the determination of rare alleles by mutation-selection balance.

A similar conclusion was made by Allendorf *et al.* (1982) after finding a mean null allele frequency of 0.0031 (29 loci) for *Pinus ponderosa* and 0.0028 (27 loci) for *P. resinosa* (Table 1.6). In contrast to Voelker and Langley's screen method the procedure used by Allendorf *et al.* (1982) was less complex due to the reproductive biology of gymnosperms. To assign a diploid genotype to each tree eight megagametophytes were analysed by electrophoresis; a null allele present in this haploid tissue was apparent by a lack of activity after staining for the enzyme in question. There were some differences between the two pine species in the distribution of null alleles, thought to be due to the differences in sample sizes but, in general, nulls appeared to be rare being present at frequencies consistent with maintenance by mutation-selection balance. However, Allendorf *et al.* (1982) were surprised at the high frequency of nulls found at the *Dia-2* locus of *P. resinosa* (0.059), but nevertheless maintained that there was no tendency

in their data for a higher frequency of nulls to occur in enzymes encoded by multiple loci; *Dia-2* is one of three diaphorase loci in this pine.

Voelker *et al.* (1980a) failed to find a correlation between null allele frequency and enzyme subunit weight, a surprising observation (as Voelker and co-workers note) since the greater the size of a subunit, the greater the number of nucleotide pairs that can mutate to give an altered structure and/or function. Allendorf *et al.* (1982) were unable to make the same comparison as the enzyme subunit molecular weights of the pine species were unknown, but there was a tendency in their data for less variable loci to have a higher frequency of null alleles. The authors felt this was unexpected given the reported positive correlation between subunit molecular weights and allozymic heterozygosity in other organisms and suggest that selection rather than mutation rate is the primary factor determining the frequency of null alleles.

Nevertheless, it is possible that some loci will have higher mutation rates than others. Tobari and Kojima (1972) found different mutation rates of electrophoretic variants for the *Idh* (3.4×10^{-5}) and the *Gpdh* (1.1×10^{-5}) loci of *D. melanogaster*. Voelker, Schaffer, and Mukai (1980b) in a study of the *D. melanogaster l(AW)* and *l(JH)* lines of Mukai and Cockerham (1977) found that the spontaneous mutation rate to null alleles of *Gpdh* was nearly twice that of *Hex-C* and seven times that found for *Got*-2.

In the earlier study, Mukai and Cockerham (1977) estimated an average rate of spontaneous mutation to null alleles of 1.03×10^{-5} over five loci while Voelker *et al.* (1980b) estimated an average rate of 3.86×10^{-6} over seven loci. This was higher than their estimate of 1.28×10^{-6} for new electrophoretic mobility variants which were obtained from the same study.

Coyne and Felton (1977), in noting a higher frequency of null alleles at the *Adh-6* locus in populations of *D. pseudoobscura* and *D. persimilis*, have suggested that nulls may be produced more frequently than other variants. Mohrenweiser (1981) has also observed a higher frequency of null mutations in comparison to mobility variants in human erythrocyte enzymes. In a study of nine enzymes, Mohrenweiser (1981) calculated the frequency of enzyme deficiency variants to be 0.0024 in newborn infants, 2 to 3 times the frequency observed by Neel, Mohrenweiser, and Meisler (1980) for rare electrophoretic variants in the same sample.

The higher mutation rate to null alleles as compared to other electrophoretic variants, both in *D. melanogaster* and humans, may reflect the greater probability for mutations to disrupt gene expression over mutations altering electrophoretic phenotypes. This may be a function of the type of mutations responsible or the structure of the enzymes in question. Alternatively, null alleles may just be easier to detect with a large proportion of the mobility variation being cryptic or undetectable under the electrophoretic conditions used. Whatever the reason, it is clear that the mechanisms responsible are acting in a similar fashion both in humans and in *D. melanogaster*. In fact, Neel (1983) has noted the similarity in mutation rates between *Drosophila*, mice, and humans despite the major differences in generation time, reproductive biology, and general metabolism. Certainly, the null frequency of 0.0024 for human erythrocyte enzymes is consonant with those reported by Voelker *et al.* (1980a), Langley *et al.* (1981), and Allendorf *et al.* (1982). Overall, these null allele frequencies are compatible with the mutation rate to null alleles of 3.86×10^{-6} estimated by Voelker *et al.* (1980b) which, to date, probably represents the best approximation to null mutation rates in natural populations.

In conclusion, there are four general points which can be made concerning the frequency and occurrence of null alleles at allozyme loci in natural populations. First, null alleles are found in a wide variety of plant and animal species and do not appear to be associated with any particular taxon, ecological niche, metabolism, or population structure. Second, the frequency of null alleles in natural populations appears to depend on the organism, the enzyme in question and the number of loci encoding for that enzyme. Null alleles occur in a wide range of enzymes, but are often present at very high frequencies at esterase loci and there is some evidence indicating an association with the non-glucose-metabolising or Group II enzymes. In addition, if there are several loci encoding for the same enzyme, nulls may occur more frequently or if duplicate genes exist, then null alleles may be fixed at one of the two loci.

Third, a danger with interspecific comparisons of null frequencies is the possibility of local population mutator activity. Genetic factors influencing mutation rates have been well documented in natural populations of *D. melanogaster* (for a review see Woodruff, Slatko, and Thompson, 1983) and there is some recent evidence for similar phenomena in mice and perhaps in humans (Neel, 1983).

Fourth, surveys of human, *Pinus* and *Drosophila* populations have provided consonant results supporting the general assumption that null alleles are infrequent at allozyme loci in natural populations. Some of the frequencies reported by the Voelker, Langley, and Allendorf surveys were polymorphic at the 1% or 5% level, but many of the nulls reported by Voelker *et al.* (1980a) and Langley *et al.* (1981) have since been identified as low activity variants (Williamson, 1982; Bentley, Meidinger, and Williamson, 1983; Burkhart, Montgomery, Langley, and

Voelker, 1984; Dr J.B. Gibson, *personal communication*). This could also apply to many of the reports reviewed in this Chapter as few studies isolate the null alleles and very few characterise them in any detail. Speculation is often common as to the possible cause of a null mutation, but to my knowledge, possibly with the exception of some human deficiencies, no studies have sufficiently characterised an allozyme null allele from a natural population to determine the mutation responsible.

The discovery of *Adh* null alleles at relatively high frequencies in Australian *D. melanogaster* populations is surprising in the light of the evidence from this review as in *D. melanogaster* there is only one *Adh* gene.

1.2 The Alcohol Dehydrogenase Polymorphism in *D. melanogaster*

The *Adh* locus is located on the second chromosome of *D. melanogaster* at map position 50.1 and is situated within the polytene chromosome bands 35B1-5, most probably within 35B2 (Grell, Jacobson, and Murphy, 1965; O'Donnell, Mandel, Krauss, and Sofer, 1977; Woodruff and Ashburner, 1979). Electrophoretic variation at the *Adh* locus was first reported by Johnson and Denniston (1964) and since then, worldwide reports have observed many natural populations to be polymorphic for several variants (O'Brien and MacIntyre, 1969; Berger, 1971; Vigue and Johnson, 1973; Voelker, Cockerham, Johnson, Schaffer, Mukai, and Mettler, 1978; Oakeshott, Gibson, Anderson, Knibb, Anderson, and Chambers, 1982). Most natural populations sampled are polymorphic for two common alleles, one encoding an allozyme of fast electrophoretic mobility (Adh^F) and one of slow mobility (Adh^S). However, several other mobility and thermostability variants have been found in natural

populations; they are generally rare, but sometimes at polymorphic frequencies. Some examples include: Adh^{UF} an ultrafast mobility variant from Spain (Malpica and Briscoe, unpublished, cited by Chambers, Wilks, and Gibson, 1984), Adh^{Ss} and Adh^{Fr} alleles which produce a slow-mobility heat-sensitive and a fast-mobility heat-stable allozyme respectively (Sampsell, 1977) and the variant $Adh^{FCh.D.}$ isolated from an Australian population which encodes a heat stable allozyme of fast mobility (Gibson, Chambers, Wilks, and Oakeshott, 1980).

The ADH enzyme (EC. 1.1.1.1) of *D. melanogaster* is a dimer composed of two identical subunits, each of 254 amino acids and a molecular weight of about 25,000 to 27,400 (Schwartz, Gerace, O'Donnell, and Sofer, 1975; Thatcher, 1980; van Delden, 1982). The ADH-F and ADH-S allozymes have been sequenced and differ by a single amino acid substitution at position 192; ADH-S differs from ADH-F by the replacement of threonine with lysine (Fletcher, Ayala, Thatcher, and Chambers, 1978; Retzios and Thatcher, 1979). Some of the less common ADH allozyme variants have also been reported to be due to single amino acid substitutions; Thatcher (1980) found that ADH-UF differed from ADH-S by the replacement of alanine by asparagine at position 45 in addition to the difference at position 192 while Chambers, Laver, Campbell, and Gibson (1981) reported a proline-214 to serine substitution between ADH-F and ADH-FCh.D..

ADH in *D. melanogaster* is present at peak levels in the third instar larvae and in six to eight day old adults. At these times it comprises up to 1% of the total soluble protein (Benyajati, Wang, Reddy, Weinberg, and Sofer, 1980). ADH activity is primarily confined to the intestine, fat body, and Malipighian tubules although in adult male flies high specific activities are found in parts of the genital apparatus (van Delden, 1982). Larvae and adult flies homozygous for the

Adh^F allele possess greater *in vitro* activity than *Adh*^{S} homozygotes with *Adh*^{F}/*Adh*^{S} heterozygotes having intermediate activities (Rasmuson, Nilson, Rasmuson, and Zeppezauer, 1966; Gibson, 1970; Day, Hillier, and Clarke, 1974). However, within each electrophoretic class there can be considerable variation in the level of ADH activity (Ward, 1975; Lewis and Gibson, 1978). This variation may be due to the action of regulatory or modifier loci as reported by Thompson, Ashburner, and Woodruff (1977), Maroni and Laurie-Ahlberg (1983) and McDonald and Ayala (1978) or to the effects of environmental factors. For example, Clarke and Whitehead (1984) have shown that the amount of yeast present in culture media is critical to the quantity of ADH.

In contrast to the data concerning activity, the amount of ADH protein in *Adh*^{F} and *Adh*^{S} homozygous strains has, in the past, been the subject of some debate (see Gibson and Miklovich, 1971; O'Brien and MacIntyre, 1978; or Clarke, 1975). It is now generally accepted that *Adh*^{F} strains have more ADH molecules at equilibrium than *Adh*^{S} strains (Gibson and Miklovich, 1971; Lewis and Gibson, 1978; van Delden, 1982; Anderson and McDonald, 1983).

Grell, Jacobson, and Murphy, (1968) first reported that ADH-negative flies died in the presence of ethanol and since then the biological role of ADH has been taken to be the detoxification of environmental alcohol. More recently, Heinstra, Eisses, Schoonen, Aben, de Winter, van der Horst, van Marrewijk, Beenakkers, Scharloo, and Thorig (1983) have proposed that ADH is not only involved in the oxidation of ethanol to acetaldehyde, but can additionally catalyse the conversion of this highly toxic product into acetate. This proposal has been challenged by Garcin, Larochelle, Lau You Hin, and Cote (1985) who believe that another enzyme, acetaldehyde dehydrogenase, is the major enzyme involved in the second reaction.

Clinal distributions of the two common *Adh* variants have been reported both in the Northern and Southern Hemispheres (Pipkin, Rhodes, and Williams, 1973; Vigue and Johnson, 1973). Wilks, Gibson, Oakeshott, and Chambers (1980), Anderson (1981), and Oakeshott *et al.* (1982) have surveyed natural populations spanning 30` of latitude in Australasia and found a significant latitudinal cline with the frequency of Adh^S decreasing with increasing distance from the equator. From Wilks *et al.* (1980) the Adh^S frequency changes from 0.94 for the most northern population sampled in Papua New Guinea to 0.49 in Southern Tasmania. Of the 34 populations sampled, 19 had the $Adh^{FCh.D.}$ allele present, but never at frequencies exceeding 0.06. It is worth noting that the Adh^S cline in Tasmania is reversed with northern Tasmanian populations having lower frequencies (Anderson and Gibson, 1985). This reversal in the cline is not paralleled in samples from comparable latitudes in New Zealand (Gibson and Freeth, unpublished data).

The *Adh* locus lies close to the proximal breakpoint of the inversion *In(2L)t* which also shows a clinal distribution decreasing in frequency with distance from the equator (Knibb, Oakeshott, and Gibson, 1981). However, the Adh^S frequency cline was found to persist in the standard chromosomes and Knibb (1983) concluded that *In(2L)t* could only account for a small fraction of the *Adh* latitudinal cline in Australasia.

In analyses of published data, Oakeshott *et al.* (1982) have confirmed that the Adh^S latitudinal cline exists in Asia, Europe, and North America. These analyses showed that for all these continents (as well as Australasia) the variation in Adh^S frequency was positively correlated with maximum rainfall for the wettest month of the year. This correlation accounted for about half of the latitudinal variation

observed. As noted by Gibson (1982), the ecological relevance for such an association is uncertain given the difficulty in relating rainfall directly to the different properties of the ADH allozymes. However, the occurrence of clinal patterns on four continents and in each case their positive association with rainfall has been taken as strong evidence that the electrophoretic variation at the *Adh* locus, or the surrounding genetic landscape, is maintained by some mode of natural selection (Gibson, 1982).

In Europe the cline in *Adh* allele frequency is associated with a cline in ethanol tolerance (David and Bocquet, 1975; Anderson, 1982). In testing for similar associations in Australian populations, Anderson (1982) found a cline in ADH activity explainable by the observed cline in *Adh* allele frequency. However, a cline in ethanol tolerance was observed which was largely independent of the *Adh* gene-enzyme system.

Indeed, the relationship between ADH activity and ethanol tolerance has been a matter of some controversy (see Gibson and Oakeshott, 1982 and van Delden, 1982 for reviews). Early studies on inbred lines, or on material which had been in the laboratory for several generations, did establish a connection between ADH activity and ethanol tolerance as expected from the observed mortality of ADH-negative flies on ethanol-containing food. These single- or multi-generation experiments on ethanol supplemented media showed an increase in Adh^F frequency, although not invariably to fixation (see review in Oakeshott and Gibson, 1981). However, similar experiments using outbred or freshly captured material have not shown an increase in Adh^F frequency (Oakeshott, Wilson, and Gibson, 1983; Oakeshott, Gibson, and Wilson, 1984).

Several studies on Australian winery populations have supported the genetic independence of ethanol tolerance and ADH activity. Two *D. melanogaster* populations located in Victoria at the wineries 'Chateau Tahbilk' and 'All Saints' have been extensively studied. Consonant results have been found for both populations in terms of species distribution, ethanol tolerance and *Adh* allele frequency. The sibling species of *D. melanogaster, D. simulans* has lower levels of ADH activity than Adh^S homozygotes and a much lower ethanol tolerance, and it is rare inside both wineries (McKenzie and Parsons, 1972; McKenzie, 1974; Gibson, May, and Wilks, 1981). McKenzie and Parsons (1974) and McKenzie and McKechnie (1978) have shown that within the vineyard, the *D. melanogaster* most closely associated with alcohol in the environment (i.e., in the vineyard cellar) were more tolerant to ethanol than those collected outside. However, this adaptation appeared to be independent of the ADH system as no significant differences were found in Adh^F allele frequencies between the cellar and outside areas.

At the All Saints winery Gibson and Wilks (1986) have located three breeding habitats of progressively decreasing ethanol concentrations. Inside the winery, leakages from barrels of fortified wines contain a mean ethanol concentration of 3.16% (v/v), while at fermentation vats near the winery entrance and at a grape skin dump some 500m from the winery ethanol concentrations are lower (2.17% and 1.26% respectively). Gibson and Wilks (1986) found that the ethanol tolerance of wild caught *D. melanogaster* males paralleled the levels of ethanol at the three breeding sites, but *Adh* alleles and ethanol tolerance segregated largely independently of one another. The evidence indicated that the frequencies of *Adh* alleles did not change with adaptation of the populations to different ethanol levels, in agreement with the results of McKenzie and McKechnie (1978).

These findings were contrary to the reports of Briscoe, Robertson, and Malpica (1975) and Hickey and McLean (1980) who reported differences in Adh^F allele frequencies between winery and neighbouring non-winery *D. melanogaster* populations from Spain and Canada. Although arguments of distance and gene flow between breeding sites have been invoked to explain this discrepancy, it is now generally accepted that although ADH may play a role in ethanol tolerance, other loci independent of the *Adh* locus are involved (Gibson and Oakeshott, 1982; van Delden, 1982; Kerver and van Delden, 1985).

Middleton and Kacser (1983) have examined the *in vivo* action of ADH by measuring the metabolic flux in the conversion of ethanol to the two products of CO_2 and lipids. Homozygote and heterozygote genotypes containing either the two common active alleles, Adh^F and Adh^S, or an artificially-induced *Adh* null allele were tested. Apart from the *Adh* null (Adh^n) homozygote, all active genotypes (including the Adh^F/Adh^n and Adh^S/Adh^n heterozygotes) showed no significant differences in metabolic flux despite the large range of *in vitro* enzyme activity. In addition, all the genotypes showed the same survivorship on ethanol supplemented media except the *Adh* null homozygote strain which suffered a high mortality consistent with its negligible metabolic flux. They conclude that the *in vitro* differences in ADH activity between Adh^F/Adh^F and Adh^S/Adh^S homozygotes are unlikely to be important in fitness differences between genotypes, at least as far as ethanol is concerned.

Other studies have also found a high mortality for *Adh* null strains on ethanol media (Grell *et al.*, 1968; Vigue and Sofer, 1976; David, Bocquet, van Herrewege, Fouillet, and Arens, 1978). In laboratory populations polymorphic for an *Adh* null allele (initial frequency 0.5) and either the Adh^F or the Adh^S allele, complete loss of the null allele

was observed on ethanol medium within eight generations (van Delden and Kamping, 1979, cited by van Delden 1982). In contrast, on media containing 1-pentene-3-ol, an alcohol converted to a poisonous ketone by ADH (Sofer and Hatkoff, 1972), a rise in the frequency of the *Adh* null allele was observed (van Delden, 1982).

The null alleles used in these and similar experiments have been derived by ethyl methanesulfonate, formaldehyde, or X-ray mutagenesis (Gerace and Sofer, 1972; O'Donnell *et al.*, 1977; Aaron, 1979). Generally the purpose of the mutagenesis was to obtain mutants for investigations of the control and structure of the *Adh* locus (see Schwartz and Sofer, 1976; Ashburner, Camfield, Clarke, Thatcher, and Woodruff, 1979; Benyajati, Place, and Sofer, 1983).

In contrast, this thesis describes the discovery and characterisation of *Adh* null alleles isolated from natural populations of *D. melanogaster*. The discovery of the *Adh* null alleles in the Australian populations was unexpected and the initial work concentrated on the estimation of their frequency in these populations. Following this, the characterisation of the isolated *Adh* null alleles was concerned first with determining if any ADH protein was produced by these alleles and second with identifying the type of mutation responsible for the null phenotype.

Table 1.1. Summary of enzymes from investigations of natural populations reporting the occurrence of null alleles († includes indophenol oxidase and superoxide dismutase).

Enzyme	Abbreviation	EC number	Number of investigations	Number of investigations with evidence of a null allele
Acid phosphatase	ACPH	3.1.3.2	8	6
Adenylate kinase	AK	2.7.4.3	1	0
Alcohol dehydrogenase	ADH	1.1.1.1	7	4
Aldehyde oxidase	AO	1.2.3.1	1	1
Aldolase	ALD	4.1.2.13	3	1
Alkaline phosphatase	ALP	3.1.3.1	1	0
Aminopeptidase	AP	3.4.11.11	1	1
Amylase	AMY	3.2.1.-	1	1
Creatine kinase	CK	2.7.3.2	1	0
Diaphorase	DIA	1.6.4.3	1	1
Esterase	EST	3.1.1.-	25	23
Fumarase	FUM	4.2.1.2	3	0
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	4	0
Glutamate dehydrogenase	GDH	1.4.1.2	2	1
Glutamate oxaloacetate transaminase	GOT	2.6.1.1	7	3
Glutamate pyruvate transaminase	GPT	2.6.1.2	1	0
Glycerol-3-phosphate dehydrogenase	GPDH	1.1.1.8	4	1
Hexokinase	HEX	2.7.1.1	2	1
Hexose-6-phosphate dehydrogenase	HPD	-	1	0
Isocitrate dehydrogenase	IDH	1.1.1.42	5	3
Lactate dehydrogenase	LDH	1.1.1.-	3	0
Leucine aminopeptidase	LAP	3.4.11.1	9	6
Malate dehydrogenase	MDH	1.1.1.37	10	4
Malic enzyme	ME	1.1.1.40	5	2
Mannosephosphate isomerase	MPI	5.3.1.8	2	1
Octanol dehydrogenase	ODH	1.1.1.73	5	2
Peptidase	PEP	3.4.-.-	3	1
Peroxidase	PER	1.11.1.7	2	1
Phosphoglucomutase	PGM	2.7.5.1	9	2
6-Phosphogluconate dehydrogenase	PGD	1.1.1.44	5	1
Phosphoglucose isomerase	PGI	5.3.1.9	8	4
3-Phosphoglycerate kinase	PGK	2.7.2.3	1	0
Sorbitol dehydrogenase	SDH	1.1.1.14	2	0
Tetrazolium oxidase†	TO	-	5	0
Triosephosphate isomerase	TPI	5.3.1.1	1	0
Xanthine dehydrogenase	XDH	1.2.3.2	3	1

Table 1.2. Compilation of electrophoretic population studies of invertebrate and vertebrate species with evidence of a null allele. (D = heterozygote deficiency, H = null homozygote phenotype, S = segregation tests, - = no information).

Organism	Common name	Enzyme	Number of loci	Evidence for a null allele	Estimates of null allele frequency	Reference
Mytilus edulis†	blue mussel	LAP	-	D, H	0.001	Koehn and Mitton, 1972
Mytilus edulis†	blue mussel	LAP	-	D, H	0.07 - 0.16	Boyer, 1974
Mytilus edulis†	blue mussel	†††	-	D, H	0.003 - 0.083††	Skibinski *et al.*, 1983
Mytilus edulis	blue mussel	LAP	2	S	-	Mallet *et al.*, 1985
Arion ater†	slug	EST(α)††††	4	H	-	Burnet, 1972
Gammarus oceanicus	marine amphipod	PGI	1	S	0.007	Siegismund, 1985b
Gammarus oceanicus	marine amphipod	MPI	1	D, S	0.01	Siegismund, 1985b
Gammarus palustris†	salt marsh amphipod	AMY	1+	D, H	0.055 - 0.23	Borowsky *et al.*, 1985
Emerita talpoida†	sand crab	PGI	-	H	-	Corbin, 1977
Mus musculus	house mouse	EST(α)	4+	D, H	0.08 - 0.39††	Selander and Yang, 1969
Calomys musculinus	rodent	EST	6	H	0.36	Gardenal *et al.*, 1980
Microtus agrestis†	field vole	EST(α,β)	-	H, S	0.22 - 0.59	Semeonoff and Robertson, 1968

†more than one population (or locality) investigated.

††estimates from more than one loci.

†††AP, EST, GOT, IDH, LAP, PGI, and PGM.

††††alpha- or beta-naphthylacetate substrate specificity is indicated for the locus (or loci) at which the null allele was detected.

Table 1.3. Plant species in which population studies have found evidence for a null allele. Abbreviations and footnotes as for Table 1.2.

Plant	Common name	Enzyme	Number of loci	Evidence for a null allele	Estimates of null allele frequency	Reference
Picea abies†	-	ACPH	-	H	0.15 - 0.167	Tigerstedt, 1973
Picea abies†	-	LAP	2	H	0.05 - 0.073	Tigerstedt, 1973
Lycopersicon cheesmanii†	Galapagos tomato	ACPH	2	H, S	-	Rick and Fobes, 1975
Lycopersicon cheesmanii†	Galapagos tomato	GOT	4	H, S	-	Rick and Fobes, 1975
Lycopersicon cheesmanii†	Galapagos tomato	PER	7	H, S	-	Rick and Fobes, 1975
Stephanomeria exigua†	-	ACPH	1+	H, S	0.01 - 0.12	Gottlieb, 1975
Stephanomeria exigua†	-	EST	3+	H, S	0.01 - 0.40††	Gottlieb, 1975
Hordeum sp.†	barley	EST	4	H, S	0.01 - 0.40††	Kahler and Allard, 1981
Oryza sativa†	rice	ACPH	2	H, S	-	Chern and Katayama, 1982
- †	Ethiopian barley	ACPH	-	H	0.023 - 0.704	Bekele, 1983
- †	Ethiopian barley	EST	4	H	0.019 - 1.0††	Bekele, 1983
Setaria italica†	millet	EST(α)	2	S	-	Kawase and Sakamoto, 1984
- †	maize	MDH	6+	H, S	-	Goodman *et al.*, 1980

Table 1.4. Insect species in which population studies have found evidence for a null allele. Abbreviations and footnotes as for Table 1.2.

Insect	Common name	Enzyme	Number of loci	Evidence for a null allele	Estimates of null allele frequency	Reference
Colias eurytheme†	sulphur butterfly	EST(α)	3	H, S	-	Burns and Johnson, 1967
Aphis pomi†	apple aphid	EST(β)	2	H	-	Singh and Rhomberg, 1984
Aedes aegypti†	mosquito	MDH	-	-	0.016 - 0.036	Gubler *et al.*, 1982
Aedes aegypti†	mosquito	ODH	-	-	0.009	Gubler *et al.*, 1982
Anopheles punctipennis	mosquito	EST	6	H, S	-	Narang and Kitzmiller, 1971
Culex pipiens pipiens†	mosquito	EST(α,β)	2	H, S	0.018 - 0.835††	de Stordeur, 1976
Drosophila athabasca†	-	EST	-	-	0.01 - 0.06	Johnson, 1978
Drosophila athabasca†	-	XDH	-	-	0.01 - 0.02	Johnson, 1978
D. cardinoides	-	EST	-	-	0.023	Napp and Cordeiro, 1978
D. dunni	-	EST(α,β)	5	H, S	-	Carrasco *et al.*, 1984
D. melanogaster†	vinegar fly	EST	-	S	-	Cambissa *et al.*, 1982
D. montana†	-	EST(α)	4	S	0.361 - 0.768††	Baker, 1975
D. neocardini itambacuriensis†	-	EST	-	-	0.453	Napp and Cordeiro, 1978
D. persimilis†	-	ADH	-	S	-	Coyne and Felton, 1977
D. polymorpha†	-	EST	-	-	0.013 - 0.020	Napp and Cordeiro, 1978
D. pseudoobscura†	-	ADH	8	-	0.014 - 0.167	Singh, 1976
D. pseudoobscura†	-	EST	-	-	0.007 - 0.03	Prakash, 1974
D. willistoni†	-	-	-	-	-	Ayala *et al.*, 1972
Dacus oleae†	olive fruit fly	EST	2	S	-	Zouros *et al.*, 1968

Table 1.5. Frequencies of null alleles at 25 enzyme loci in a Raleigh (North Carolina) and a London (England) population of *D. melanogaster* as reported by Dr R.A. Voelker, Dr C.H. Langley and co-workers. (Table modified from Voelker *et al.*, 1980a and Langley *et al.*, 1981).

Enzyme locus	Genetic map position	North Carolina		England	
		Number of alleles scored	Null allele frequency	Number of alleles scored	Null allele frequency
Pgd	1 - 0.6	799	0.000	496	0.000
Fum	1 - 19.9	770	0.000	502	0.000
Hex-A	1 - 29.2	760	0.000	497	0.000
Gpt	1 - 42.6	794	0.000	493	0.000
G6pd	1 - 63	737	0.000	491	0.000
Got-2	2 - 3.0	782	0.004	357	0.000
Pgk	2 - 5.9	702	0.000	429	0.000
Gpdh	2 - 17.8	814	0.009	386	0.008
cMdh	2 - 37.2	815	0.002	408	0.000
Adh	2 - 50.1	808	0.001	362	0.000
Pep-A	2 - 55.2	767	0.003	380	0.008
Pgi	2 - 58.6	716	0.001	436	0.000
Hex-C	2 - 73.5	796	0.001	369	0.003
Idh	3 - 25.4	916	0.001	450	0.004
Est-6	3 - 36.0	804	0.000	418	0.000
Pgm	3 - 43.4	913	0.000	431	0.002
Est-C	3 - 47.9	758	0.005	408	0.005
Odh	3 - 49.2	769	0.001	404	0.000
Me	3 - 51.73	734	0.008	413	0.002
Xdh	3 - 52.0	575	0.000	401	0.000
Ao	3 - 56.7	739	0.012	413	0.010
mMdh	3 - 62.6	723	0.000	450	0.000
Ald	3 - 91.5	912	0.000	438	0.000
Acph-1	3 - 101.1	799	0.001	418	0.005
Tpi	3 - 101.3	637	0.000	426	0.000

Table 1.6. Null allele frequencies at 31 enzyme loci in two *Pinus* species as reported by Dr F.W. Allendorf and co-workers (Table modified from Allendorf *et al.*, 1982).

Enzyme locus	*P. ponderosa*		*P. resinosa*	
	Number of alleles scored	Null allele frequency	Number of alleles scored	Null allele frequency
Adh	792	0.000	221	0.005
Ak	724	0.000	-	-
Ald	760	0.001	221	0.000
Ck	-	-	48	0.000
Dia-1	732	0.000	193	0.000
Dia-2	662	0.000	221	0.059
Dia-3	758	0.001	221	0.000
Gdh	798	0.006	221	0.000
Got-1	792	0.000	221	0.000
Got-2	702	0.000	221	0.000
Got-3	764	0.000	221	0.000
G6pd-2	764	0.000	221	0.000
Idh	800	0.003	221	0.000
Lap-1	534	0.000	221	0.000
Lap-2	788	0.017	-	-
Mdh-1	794	0.021	221	0.000
Mdh-2	790	0.006	221	0.000
Mdh-3	784	0.000	221	0.000
Mdh-4	796	0.000	221	0.000
Me	786	0.034	221	0.000
Mpi	790	0.000	-	-
Pep-1	788	0.000	221	0.000
Pep-2	542	0.000	221	0.000
Pep-3	-	-	221	0.000
Pgd-1	794	0.000	221	0.000
Pgd-2	798	0.000	221	0.014
Pgi-1	786	0.000	221	0.000
Pgi-2	788	0.000	221	0.000
Pgm-1	170	0.000	-	-
Pgm-2	786	0.000	-	-
Sdh	790	0.000	221	0.000

Table 1.7. Occurrence of null alleles at Group I and Group II allozyme loci (designations as per Kojima *et al.*, 1970) listed as a fraction of the number of investigations in which null alleles were detected/total number of investigations for that enzyme. Abbreviations as in Table 1.1.

GROUP I		GROUP II	
Glucose-metabolising enzymes		Non-glucose-metabolising enzymes	
ALD	1/3	ACPH	6/8
FUM	0/3	ADH	4/7
G6PD	0/4	AO	1/1
GPDH	1/4	ALP	0/1
HEX	1/2	EST	23/25
IDH	3/5	ODH	2/5
MDH	4/10	XDH	1/3
ME	2/5		
PGM	2/9		
PGD	1/5		
PGI	4/8		

Table 1.8. Occurrence of null alleles at variable-substrate, regulatory, and non-regulatory enzyme systems (designations as per Powell, 1975), listed as a fraction of the number of investigations in which null alleles were detected/total number of investigations for that enzyme. Abbreviations as in Table 1.1.

Variable-substrate enzymes		Regulatory enzymes		Non-regulatory enzymes	
ACPH	6/8	AK	0/1	ALD	1/3
ALP	0/1	ADH	4/7	AMY	1/1
EST	23/25	HEX	1/2	FUM	0/3
LAP	6/9	ME	2/5	G6PD	0/4
ODH	2/5	PGI	4/8	GPDH	1/4
PEP	1/3	PGM	2/9	GOT	3/7
TO	0/5	XDH	1/3	LDH	0/3
				MDH	4/10
				PGD	1/5
				SDH	0/2
				TPI	0/1

CHAPTER TWO

DISCOVERY AND CONFIRMATION OF NULL ALLELES

2.1 Introduction

In 1983 a series of collections were made by Dr J.B. Gibson and Ms A.V. Wilks from Tasmanian *D. melanogaster* populations. The main aims were first to assay *Adh* and *Gpdh* gene frequencies over the island as there was evidence of a reversal to the reported *Adh* cline (Anderson and Gibson, 1985), and second to isolate a number of homozygous lines.

During the analysis of these samples a number of anomalies were detected in isofemale cultures established from wild-caught females. Among the alternatives, the most likely explanation for these anomalies appeared to be the presence of a null allele(s). This Chapter describes the initial detection of the anomalous cultures, the null allele proposal and the subsequent experiments and isolation programme which verified this hypothesis.

2.2 Materials and Methods

2.2.1 Collections

D. melanogaster populations were sampled in May 1983 in Tasmania by Dr J.B. Gibson and Ms A.V. Wilks. Two sites in the south of Tasmania, Cygnet and Huonville (I), are apple processing factories about 10km apart and two sites 300km to the north, Tamar Valley and Pipers Brook, are wineries (30km apart). At Cygnet and Huonville (I) flies were breeding on decomposing apple pulp while collections at Tamar and Pipers Brook were made over piles of discarded grape pressings.

At all sites adult flies were net swept and transported on standard laboratory media described in Section 2.2.2. The populations of adult flies were very large at all sites.

From each of these samples between 66 and 81 wild-caught fertilised females were set up singly in vial cultures. Each female parent was

classified by electrophoresis for ADH and GPDH allozymes (Section 2.2.3). In addition, ten F1 progeny of each female parent were also classified in order to infer the electrophoretic phenotype of the unknown male mate. In a two allele system classification of ten progeny will allow the correct determination of a wild mating with a probability of 0.998, if the female parent is a homozygote, and a probability of 0.888 if she is a heterozygote (Barrett, 1977).

To compare with the Tasmanian samples wild-caught flies from a Coffs Harbour (N.S.W.) banana plantation were treated in the same way. These flies which were breeding on piles of decomposing bananas and avocados had been caught by Drs I. Boussy and S. Easteal.

2.2.2 *Drosophila* culture

All *Drosophila* stocks and experimental crosses were routinely cultured in glass vials or 250ml plastic bottles at 22 to 25°C on a standard media composed of agar (10g), glucose (50g), maize meal (50g), sucrose (26g), wheat germ (22.5g), and yeast (6g) in 1 litre of water with 12.5ml of acid mix (42% propionic acid, 4% orthophosphoric acid). Some single pair crosses were made on a double yeast media (12g instead of 6g) or on high protein food (agar [10g], corn syrup [30ml], high protein powder [HiPro, 10g], malt [40g], sucrose [15g] and yeast [35g] in 1 litre of water with 4.5ml of propionic acid), but cultures involved with ADH activity measurement were always maintained on the standard yeast media.

2.2.3 Electrophoresis

Electrophoresis for ADH was as described by Lewis and Gibson (1978) and for GPDH by Gibson, Wilks, Cao, and Freeth (1986). ADH and GPDH phenotypes were scored after electrophoresis of single fly homogenates on cellulose acetate membranes (Chemetron). Membranes were soaked in

running buffer for at least 15 minutes before use for both ADH (35mM Tris, pH8.8, 5mM boric acid, 3.5mM EDTA) and GPDH (126mM Tris, pH6.6, 42mM citric acid, 9.26mM EDTA). Electrophoresis was for 30 to 45 minutes at 4mA (300V) for ADH and 8mA (480V) for GPDH. Staining for ADH activity was in 50mM orthophosphate buffer (pH7.8) using a tetrazolium salt (0.5mM), isopropanol (1%), NAD (0.5mg/ml) and phenazine methosulphate (PMS, 0.01mg/ml). Membranes were stained for GPDH in 100mM Tris-HCl (pH 8.6) containing DL-glycerophosphate (7mg/ml), NAD (2mg/ml), thiazolyl blue tetrazolium salt (0.5mg/ml), PMS (0.01mg/ml) and EDTA (2mg/ml). Staining was stopped after 2 to 3 minutes by washing the membranes with water.

A spot test was devised where single fly homogenates were spotted onto the cellulose acetate membranes and stained for the enzymes as described.

2.2.4 Pentenol vapour screen

A selection technique described by Sofer and Hatkoff (1972) was used to isolate flies lacking activity. Flies are exposed to vapour of the unsaturated secondary alcohol, 1-pentene-3-ol, which is oxidised by ADH into a poisonous ketone and only those individuals which are ADH negative survive. A freshly-prepared 5% solution of 1-pentene-3-ol is placed in a 25ml flask through which air is bubbled by an aquarium pump. The resulting vapour is then directed into a half-pint milk bottle containing the flies. Male and female flies were usually tested separately. The method was slightly modified by using a 50ml flask as a trap between the 25ml flask and the milk bottle to prevent the pentenol solution from directly entering the milk bottle, and by increasing the exposure time to 4 minutes. After exposure, air was circulated through the milk bottle for 2 minutes and the surviving flies were removed and kept on standard laboratory media.

2.3 Discovery of *Adh* and *Gpdh* Null Alleles

In the following discussion phenotypes of the common 'fast' and 'slow' allozymes at the *Adh* and *Gpdh* loci are distinguished by F and S, while italicised forms of these letters represent genotypes; so individuals classified as having the electrophoretic phenotypes F, S, or FS are presumed to have the genotypes *FF*, *SS*, or *FS*. By knowing the phenotypes of the female parent and a sample of her Fl progeny the unknown male phenotype can be inferred with a known probability (Barrett, 1977; Table 2.1). This assumes that the only two alleles present at polymorphic frequencies are the *F* and *S* variants.

During the selection of the desired *FF* x *FF* and *SS* x *SS* matings from the Cygnet lines, a number of anomalous cultures were discovered where the distribution of phenotypes could not be explained by any of the mating combinations shown in Table 2.1. For example, in the 22nd isofemale line of Cygnet (C22) the female parent had been classified for ADH as having a F phenotype and assumed to be a homozygote with two copies of the *F* allele (i.e., *FF*). However, among the Fl progeny S and FS phenotypes were identified. The FS heterozygotes were explicable if the female was mated by a S (*SS*) male, but the S individuals could not be accounted for within the two allele model. Among 78 Cygnet lines 6 anomalous cultures were found, so that whatever was responsible could not be considered to be rare.

Initially two explanations were considered to account for the anomalous cultures, a mistake in phenotype classification may have occurred or, alternatively, a double mating was involved where sperm from two males of different genotypes fertilised the female. Mistakes in phenotype classification were considered unlikely due to the good separation and resolution of the ADH bands after electrophoresis.

Double mating is known to occur in natural populations of *D. melanogaster* (Milkman and Zeitler, 1974) but this proposal proved to be untenable as the progeny distributions expected were not consistent with those observed. For example, as the C22 female parent was F, then the possible mating combinations are F x F, F x S or F x FS producing the progeny phenotypes F, FS, and F and FS. Matings involving any two of these combinations could only result in F and FS phenotypes.

A third possibility was that the female parent was heterozygous for a null allele so that the original mating was between a *Fn* female and a *SS* male producing *FS* and *Sn* progeny. The *Sn* genotype could explain the S phenotypes observed. To test the hypothesis that the C22 F1 male flies with the S phenotype carried a single *S* allele, they were crossed singly to females homozygous for the allele Adh^{F71K}, which encodes a heat resistant form of ADH (Thorig, Schoone, and Scharloo, 1975; Wilks *et al.*, 1980) not found in the Tasmanian populations (Anderson and Gibson, 1985). Similarly, S males from another Cygnet line, C60, which had given the same F1 progeny distribution as C22, were crossed to Adh^{F71K} homozygotes. In both cases FS and F progeny were produced and the ADH in the F flies had the properties characteristic of ADH-F71K; for C22 49 F71K/S and 39 F71K progeny were identified while C60 produced 71 and 39 respectively. The progeny phenotypes of C22 males were in agreement with the 1:1 ratio expected ($X^2_1 = 0.92$, $p > 0.05$) while those of C60 were significantly different ($X^2_1 = 8.74$, $p < 0.01$) with a deficiency of the Adh^{F71K}/Adh^{n} genotypes. It was noticed there was a reduction in band stain intensity of F71K progeny as compared to the control Adh^{F} homozygotes run at the same time. This was expected if the F71K flies had a single *Adh* allele.

2.4 Further Tests of the Null Allele Hypothesis

Following the discovery of irregular F1 progeny phenotypes in the Cygnet samples all the isofemale lines from the three remaining Tasmanian populations were analysed for similar anomalies. As the female parents and their F1 progeny had been classified for ADH and GPDH both enzyme systems were screened for putative null alleles. In addition, the progenies of a sample of Cygnet males crossed to Canton S females were scored. Also analysed were isofemale lines from a population at Coffs Harbour set up after the discovery of the aberrant Cygnet lines. Only lines where the female had F or S phenotypes were checked, as individuals with a FS phenotype must have the genotype *FS*.

Tables 2.2 and 2.3 list all the isofemale lines in which anomalous progenies were detected. Also summarised are the presumed genotypes of both parents, and their F1 progeny together with a X^2 goodness-of-fit test of observed to expected progeny phenotypes. In total, fifteen anomalous ADH lines were detected and, except for C46, CH92, and P11, all of them had phenotype distributions in agreement with those predicted by the null allele hypothesis. Seven anomalous GPDH lines were found, of which C4, C28, and C34 had distributions significantly different to that expected by the null allele hypothesis. Low sample sizes might explain the significant X^2 values for C4, C28, C34, P11, and CH92. From line C46 30 progeny were classified, but there was a marked deficiency of the *Sn* genotype.

C46 (and C41) differed from the other lines in originating from crosses between wild-caught males and Canton S females set up as part of a hybrid dysgenesis survey by Dr I. Boussy. C41 and C46 F1 larvae and pupae were also classified for ADH phenotypes by electrophoresis. For C41, 4 FS and 16 S larvae and 6 FS and 2 S pupae were identified.

However, electrophoresis of 14 F1 C46 larvae and eight F1 pupae failed to identify any individuals with a S phenotype (i.e., all FS). The implication from this data was that the putative null allele was really an Adh^F allele which was expressed in larvae but not in adults. Unfortunately it proved impossible to isolate the variant for further study.

Taking into account the likelihood of associated differential viability effects there is, overall, general agreement between the observed and expected distribution of F1 phenotypes from the isofemale lines and this provides additional evidence supporting the null allele hypothesis.

If the genotypes assigned on the null allele hypothesis were correct, then predictions could be made about the phenotypic frequencies in the F2 generation. The following analyses are only concerned with the *Adh* lines. From selected anomalous lines a number of single F1 females were set up to produce progeny. These females could have mated with any F1 male and the progeny classes could be predicted. For example, C14 F1 females classified as S had a *Sn* genotype under the null allele hypothesis. They were free to mate with *FF*, *Fn*, *Sn*, and *FS* males giving the following progeny classes:

Fn FS

Fn Sn FS nn

SS Sn nn

Fn SS Sn FS.

Thus, individuals with phenotypes characteristic of a null homozygote (i.e., lacking ADH band activity) should be produced.

Table 2.4 lists the F1 isofemale lines as classified into mating types according to the observed progeny phenotypes. For example,

progenies were typed from nine C14 *Sn* females and of these one had mated *FF*, two *Fn*, three *Sn*, and three *FS* males. All the expected phenotypes were observed in the progenies including individuals with no ADH activity, identified as predicted in the progenies of *Sn* x *Fn* and *Sn* x *Sn* matings (Table 2.4). No homozygous null phenotypes were found for C60 and P17 possibly because in each case the null allele was borne on a homozygous lethal chromosome.

As a further test for the presence of individuals lacking ADH activity the combined progeny of some lines were exposed to the vapour of 1-pentene-3-ol. Exposure of the F2 progeny from the C14, C53, and C62 lines to pentenol vapour resulted in many flies dying, but a number of live flies were recovered. They were confirmed to be null homozygotes after electrophoresis and staining (Table 2.5). There were some significant departures from the expected genotype frequencies which could be explained by reduced viability of homozygotes.

2.5 Isolation of *Adh* Null Alleles

Adh null alleles were isolated from a number of lines (Table 2.6). None of the putative *Gpdh* null alleles were isolated at this time, although the single female lines that produced the anomalous progenies were maintained as mass cultures and subsequent work (Dr J.B. Gibson, unpublished) showed they contained low activity variants.

Isolation of the putative *Adh* null alleles was achieved by two procedures. The first used exposure to pentenol vapour to recover female and male *Adh* null homozygotes which were then mated together. In the second procedure, individuals with suitable phenotypes were crossed to a second-third chromosome translocation stock $T(2;3)\ ap^{Xa}$, which also contained the second chromosome balancer *CyO*, and the third chromosome

balancer, *TM6*. In some cases only male or only female flies survived the pentenol screen and in such cases these were then crossed to the balancer stock to isolate the null allele.

Figure 2.1 outlines the breeding scheme used to isolate the alleles using the second chromosome balancer *Cy0*. The *Cy0* chromosome was marked by an Adh^F allele and this required the putative null to be heterozygous with a *S* allele, so that the *Fn* (*Cy0*/+) and *FS* (*Cy0*/+) heterozygotes could be distinguished. A null heterozygous with a *F* allele could not be efficiently isolated as *Fn* (*Cy0*/+) and *FF* (*Cy0*/+) phenotypes are identical. If null homozygotes obtained from the pentenol screen are mated to the balancer stock, then the procedure is simplified as only *Fn* (*Cy0*/+) progeny are produced. From the original isofemale lines of C22, C41, C60, and C80 F1 male progeny were mated singly to several *T(2;3)* ap^{Xa}*/Cy0; TM6* females. The male parents were then typed for ADH and crosses in which the male had the S phenotype (see Table 2.2) were retained. From these progenies *Cy0* virgin females and males were collected and set up as single pair crosses. The parents were typed for ADH and crosses in which both parents had F phenotypes were preserved and non-Curly progeny homozygous for the null allele were collected as virgins and used to set up stocks. The wild-type third chromosome was subsequently made homozygous. As contamination of these null homozygous wild-type stocks could not be detected by visual means, pentenol vapour screening was used to test all progenies of each stock (Fig. 2.1). Null homozygotes were derived from C22 and C80, but C41 and C60 proved to be homozygous lethal and were maintained as *Cy0/n* heterozygotes.

The C14 and C53 null alleles were successfully isolated by the pentenol vapour procedure. Virgin female and male survivors from the screens described in Section 2.4 were single pair mated, confirmed to

have a null phenotype after electrophoresis, and then their progeny used to establish stocks. In addition, some remaining C14 male homozygotes were crossed to flies carrying the *CyO;TM6* balancers and a homozygous line was derived by the procedure outlined in Figure 2.1.

Progeny from the F1 isofemale lines of C62, P81, and T33, where the female parent had an F phenotype (*FF* or *Fn* genotype), were screened by pentenol vapour. Two P81 male survivors and two T33 female survivors were recovered and crossed to *CyO* and then homozygous null allele stocks were obtained. A few C62 male and female survivors were obtained, but they failed to establish a viable homozygote culture, so males were crossed to *CyO* and a *CyO/n* stock was obtained. C62 can be considered homozygous lethal as very few *n/n* individuals are produced by this stock. The putative null alleles contained in the remaining isofemale lines which produced anomalous progeny were not isolated (some were attempted, but eventually abandoned due to culture failures).

2.6 Conclusion

The isolation of *Adh* null activity homozygotes from a relatively high proportion of the isofemale lines with aberrant progeny phenotypes was surprising in view of the assumption that null alleles are rare. Both from the progeny experiments and isolation programme it seemed likely that the nulls were alleles at the *Adh* locus, but if a *cis*-acting suppressor was responsible for the loss of ADH activity then it was tightly-linked to the *Adh* locus. The apparent high frequencies of *Adh* null alleles in the Tasmanian population samples warranted further investigation.

Table 2.1. Possible F1 progeny phenotypes of homozygous females and the inferred phenotype of the male parent.

Female phenotype	F1 progeny phenotypes	Inferred male phenotype	Presumed genotype of female and male	Presumed F1 progeny genotypes
F	F	F	*FF* x *FF*	*FF*
F	F FS	FS	*FF* x *FS*	*FF FS*
F	FS	S	*FF* x *SS*	*FS*
S	S	S	*SS* x *SS*	*SS*
S	S FS	FS	*SS* x *FS*	*SS FS*
S	FS	F	*SS* x *FF*	*FS*

Table 2.2. Isofemale lines having anomalous ADH phenotype distributions in their F1 progeny (*F*, fast allele; *S*, slow allele; *n*, null allele).

Population sampled	Isofemale line	Female phenotype	F1 progeny phenotypes	Presumed genotype of female and male parents	Presumed F1 progeny genotypes	Expected ratio of F1 progeny phenotypes	Observed F1 progeny phenotypes	$\chi^2_{1 \text{ or } 2}$
						F : S : FS	F : S : FS	
Cygnet	C14	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	47 : 25 : 22	0.19
Cygnet	C22	F	S and FS	*Fn* x *SS*	*Sn FS*	0 : 1 : 1	0 : 29 : 32	0.07
Cygnet	C41	F	S and FS	*Fn* x *SS*	*Sn FS*	0 : 1 : 1	0 : 17 : 12	0.55
Cygnet	C46	F	S and FS	*Fn* x *SS*	*Sn FS*	0 : 1 : 1	0 : 5 : 25	12.03***
Cygnet	C53	S	F, S, and FS	*Sn* x *FS*	*Fn SS Sn FS*	1 : 2 : 1	21 : 38 : 16	0.68
Cygnet	C60	F	S and FS	*Fn* x *SS*	*Sn FS*	0 : 1 : 1	0 : 36 : 26	1.31
Cygnet	C62	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	39 : 19 : 20	0.03
Cygnet	C80	F	S and FS	*Fn* x *SS*	*Sn FS*	0 : 1 : 1	0 : 22 : 18	0.23
Pipers Brook	P11	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	9 : 7 : 13	6.66*
Pipers Brook	P17	S	F and FS	*Sn* x *FF*	*Fn FS*	1 : 0 : 1	16 : 0 : 16	0
Pipers Brook	P81	S	F and FS	*Sn* x *FF*	*Fn FS*	1 : 0 : 1	20 : 0 : 21	0
Tamar	T33	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	14 : 10 : 13	2.68
Coffs Harbour	CH89	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	2 : 3 : 5	4.40
Coffs Harbour	CH92	S	F, S, and FS	*Sn* x *FS*	*Fn SS Sn FS*	1 : 2 : 1	1 : 1 : 6	10.75**
Coffs Harbour	CH133	F	S and FS	*Fn* x *SS*	*Sn FS*	0 : 1 : 1	0 : 4 : 6	0.10

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 2.3. Isofemale lines having anomalous GPDH phenotype distributions in their F1 progeny (*F*, fast allele; *S*, slow allele; *n*, null allele).

Population sampled	Isofemale line	Female phenotype	F1 progeny phenotypes	Presumed genotype of female and male parents	Presumed F1 progeny genotypes	Expected ratio of F1 progeny phenotypes F : S : FS	Observed F1 progeny phenotypes F : S : FS	$\chi^2_{1 \text{ or } 2}$
Cygnet	C4	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	2 : 2 : 6	6.80*
Cygnet	C28	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	2 : 2 : 6	6.80*
Cygnet	C29	S	F and FS	*Sn* x *FF*	*Fn FS*	1 : 0 : 1	8 : 0 : 2	2.50
Cygnet	C34	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	2 : 1 : 7	10.80**
Tamar	T43	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	6 : 1 : 3	1.20
Coffs Harbour	CH48	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	3 : 2 : 2	0.14
Coffs Harbour	CH88	F	F, S, and FS	*Fn* x *FS*	*FF Fn Sn FS*	2 : 1 : 1	1 : 2 : 4	4.71

*$p < 0.05$, **$p < 0.01$

Table 2.4. Observed progeny phenotypes of F1 isofemale lines classified according to the possible matings with male sibs.

Original isofemale line†	Genotype of F1 female parent	Possible genotypes of F1 mates	Progeny phenotypes expected	F1 isofemale lines and the observed progeny phenotypes										
				1	2	3	4	5	6	7	8	9	10	11
C14	*Sn*	*FF*	F	4										
			FS	10										
C14	*Sn*	*Fn*	F	3	6									
			S	7	5									
			FS	3	1									
			n	1	2									
C14	*Sn*	*Sn*	S	10	10	10								
			n	3	4	4								
C14	*Sn*	*FS*	F	5	9	9								
			S	12	8	5								
			FS	7	6	9								
C22	*Sn*	*Sn*	S	8	18	9	5	8	7					
			n	2	2	1	5	2	3					
C22	*Sn*	*FS*	F	3	4	2	4	0	1	1	4	2	1	
			S	5	4	3	3	6	9	9	5	7	5	
			FS	2	2	5	3	4	0	0	1	1	4	
C53	*Fn*	*Fn*	F	10										
			n	4										
C53	*Fn*	*SS*	S											
			FS											
C53	*Fn*	*Sn*	F	5										
			S	5										
			FS	3										
			n	1										
C53	*Fn*	*FS*	F	8	6	11	5	9	8					
			S	6	12	7	14	4	4					
			FS	10	6	6	5	3	8					
C60	*Sn*	*Sn*	S	20	11	20	18	15	20					
			n	0	0	0	0	0	0					
C60	*Sn*	*FS*	F	0	0	0	2	2	2	0	0	1	0	2
			S	8	9	8	4	2	4	7	3	6	6	6
			FS	1	1	2	4	6	4	3	7	3	4	2

Table 2.4 continued.

Original isofemale line†	Genotype of F1 female parent	Possible genotypes of F1 mates	Progeny phenotypes expected	F1 isofemale lines and the observed progeny phenotypes										
				1	2	3	4	5	6	7	8	9	10	11
C62	*Sn*	*FF*	F	9										
			FS	5										
C62	*Sn*	*Fn*	F	3	7									
			S	4	0									
			FS	5	4									
			n	2	3									
C62	*Sn*	*Sn*	S											
			n											
C62	*Sn*	*FS*	F	6	4	10								
			S	11	11	8								
			FS	7	9	6								
P17	*Fn*	*Fn*	F	10	9	7								
			n	0	0	0								
P17	*Fn*	*FS*	F	4	5	5	7							
			S	0	0	0	0							
			FS	6	6	5	3							
P81	*Fn*	*Fn*	F	9	9	11	7							
			n	2	1	1	1							
P81	*Fn*	*FS*	F	4	3	1								
			S	3	0	1								
			FS	4	1	1								
T33	*Sn*	*FF*	F	6										
			FS	1										
T33	*Sn*	*Fn*	F	0	1	1	1							
			S	7	1	6	4							
			FS	1	5	0	6							
			n	2	1	1	4							
T33	*Sn*	*Sn*	S											
			n											
T33	*Sn*	*FS*	F	9	0									
			S	1	4									
			FS	1	11									

†C = Cygnet, P = Pipers Brook, T = Tamar.

Table 2.5. Number of *Adh* null allele homozygotes recovered after exposing the progeny of the isofemale lines of C14, C53, and C62 to pentenol vapour. Numbers represent combined totals from separate screens of female and male progeny except those indicated (†) in which only females were tested.

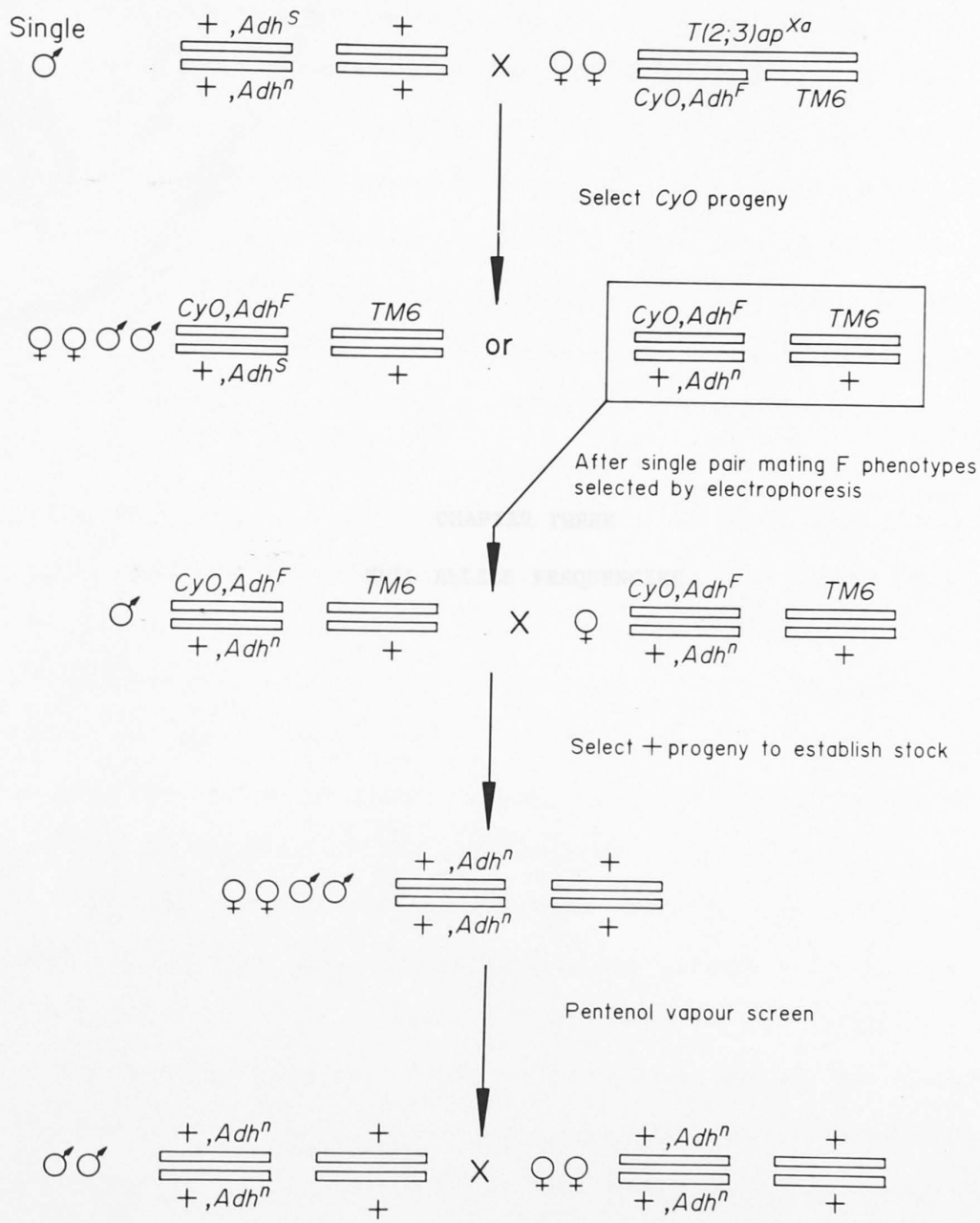
Line	Screen	Presumed genotypes of F1 females	Total number of progeny screened	Null homozygotes recovered	Number of null homozygotes expected	X_1^2
C14	1	*FF* or *Fn*	133	16	8	6.63*
	2†	*FF* or *Fn*	85	4	5	0.13
	3	*FF* or *Fn*	688	55	43	3.57
C53	1†	*SS* or *Sn*	44	1	3	0.61
	2	*SS* or *Sn*	639	22	40	8.60**
C62	1†	*FF* or *Fn*	63	3	4	0.05
	2	*FF* or *Fn*	474	16	20	6.68**

*$p < 0.05$, **$p < 0.01$.

Table 2.6. *Adh* null allele culture stocks (Y = yes, N = no).

Population from which line was established	Line from which allele was isolated	Laboratory stock(s) maintained as	Method of isolation	
			Pentenol vapour	*CyO;TM6*
Cygnet	C14	Adh^n/Adh^n	Y	Y
Cygnet	C22	Adh^n/Adh^n	N	Y
Cygnet	C41	*CyO*, Adh^F/Adh^n	N	Y
Cygnet	C53	Adh^n/Adh^n	Y	N
Cygnet	C60	*CyO*, Adh^F/Adh^n	N	Y
Cygnet	C62	*CyO*, Adh^F/Adh^n	N	Y
Cygnet	C80	Adh^n/Adh^n	N	Y
Pipers Brook	P81	Adh^n/Adh^n	N	Y
Tamar	T33	Adh^n/Adh^n	N	Y

Figure 2.1. Procedure for isolating second chromosomes carrying *Adh* null alleles using the second and third chromosome balancers *CyO* and *TM6*.


Single
+ ,Adh^S
+ ,Adh^n
+
+
X
T(2;3)ap^Xa
CyO,Adh^F
TM6
Select CyO progeny
CyO,Adh^F
+ ,Adh^S
TM6
+
or
CyO,Adh^F
+ ,Adh^n
TM6
+
After single pair mating F phenotypes selected by electrophoresis
CyO,Adh^F
+ ,Adh^n
TM6
+
X
CyO,Adh^F
+ ,Adh^n
TM6
+
Select + progeny to establish stock
+ ,Adh^n
+ ,Adh^n
+
+
Pentenol vapour screen
+ ,Adh^n
+ ,Adh^n
+
+
X
+ ,Adh^n
+ ,Adh^n
+
+

CHAPTER THREE

NULL ALLELE FREQUENCIES

3.1 Introduction

The discovery of a high proportion of anomalous cultures, shown to be due to the presence of null alleles at the *Adh* locus, in the 1983 Tasmanian samples was unexpected given the previous evidence that null alleles are rare. In the Cygnet population alone, 6 out of 78 cultures had evidence for the presence of an *Adh* null allele giving a null frequency in the females tested of 3.9%. This is 16 times the average frequency of 0.24% reported by Langley *et al.* (1981) for two *D. melanogaster* populations. These workers had estimated mean frequencies for 20 autosomal loci to be 0.25% for a Raleigh, North Carolina and 0.23% for a London, England population with a frequency at the *Adh* locus of 0.09%.

The discrepancy between the *Adh* null allele frequency in the Cygnet Australian population and that previously reported required investigation. Specifically, two questions had to be answered. First, what was the frequency of null alleles in the other Australian populations sampled in 1983? Second, did these alleles persist in the populations over time and if so, at what frequency?

Of relevance to these questions were the techniques used to detect null alleles which had differed between the present study and the two others reported in the literature. The isofemale line progeny testing procedure was originally carried out for another purpose and therefore the extent to which it detected null alleles needed to be considered. If three alleles: *F*, *S*, and *n* are present at the *Adh* and *Gpdh* loci, then six genotypes (*FF*, *Fn*, *SS*, *Sn*, *FS*, and *nn*) and 36 matings are possible (Table 3.1). The single female parents were typed, but as homozygous *nn* females were never detected matings involving these (see bottom line, Table 3.1) can be excluded.

The 20 informative matings are summarised in Table 3.2. These represent the progeny classes from which the wild-caught females can be identified to be carrying a null allele. Theoretically, the genotype of the unknown male parent can be inferred with a high probability if ten or more F1 progeny are electrophoresed (Barrett, 1977). However, a null allele present in the male parent cannot be unambiguously identified, as crosses between *SS* (or *FF*) females and *Fn* (or *Sn*) males give the same progeny phenotypes as crosses between *SS* (or *FF*) females and *FS* males (Table 3.1). Thus, in calculating the frequency of null alleles only the tested females are counted.

From Table 3.2 there are four progeny classes for either F or S female parents where a null allele can be identified. In the first two classes (see (i) and (ii), Table 3.2), a null allele is indicated by the presence of progeny with phenotypes opposite to that of their female parent. This is true of the third class which also includes null homozygote progeny and these are also produced in the fourth progeny class (see (iii) and (iv), Table 3.2). There is only one class of progeny in which a null allele, present in the female parent, remains undetectable and this occurs in lines where apparent active allele homozygotes are detected (Table 3.2). For example, if the female parent and her F1 progeny are classified as F, then (from Table 3.1) the genotypes involved in that mating could be:

(i) *FF* x *FF*

(ii) *FF* x *Fn*

(iii) *Fn* x *FF*

(iv) *FF* x *nn*

with the female in mating (iii) containing an undetectable null allele.

Assuming the population is in Hardy-Weinberg equilibrium the effect of mating (iii) can be estimated. The genotype proportions of the four matings are:

(i) $p^2.p^2$

(ii) $p^2.2pr$

(iii) $2pr.p^2$

(iv) $p^2.r^2$

where p, q and r represent the frequencies of the alleles *F*, *S*, and *n* respectively. The proportion of total matings with a *Fn* female is therefore:

$$\frac{2pr.p^2}{(p^2.p^2) + (2pr.p^2) + (p^2.2pr) + (p^2.r^2)}$$

$$= \frac{2p^3r}{p^4 + 2(2p^3r) + (p^2.r^2)}$$

$$= \frac{2pr}{p^2 + 4pr + r^2}$$

Similarly, if the female and her Fl progeny were S, then the proportion of matings with *Sn* females would be:

$$\frac{2qr}{q^2 + 4qr + r^2}.$$

Therefore, a correction for this underestimation can be applied to the null allele frequency after the calculation of *F*, *S*, and *n* allele frequencies.

As the 1983 progeny testing method used underestimated the null frequency, it seemed worthwhile in 1984 to use a procedure like the one of Voelker *et al.* (1980a) in which both alleles present in a wild-caught fly could be identified. Voelker's technique involved crosses which were potentially dysgenic (Kidwell, Kidwell, and Sved, 1977) and at least some of the alleles they detected might have arisen during the screening crosses (Burkhart *et al.*, 1984). To reduce the possibility of dysgenic phenomena homozygous tester stocks were obtained from the material isolated from the 1983 Cygnet population, and of these one was homozygous for Adh^F and $Gpdh^F$ and the other was homozygous for Adh^S and $Gpdh^S$ alleles.

Wild-caught males were mated in turn to females of both tester stocks and the two progenies were cultured separately. The male parents were typed for ADH and GPDH phenotypes and cultures derived from males heterozygous at both loci were discarded. From each set of heterogametic matings 10 flies were classified for both enzymes. Following Voelker *et al.* (1980a) the failure of the wild-caught allozyme to show normal activity as a homodimer in a heterozygote was used to designate a putative null allele. The advantage of this method over female progeny testing was that every null allele present in every male

fly tested could be identified. Table 3.3 illustrates this; *FS* heterozygotes and *nn* homozygotes are identified immediately on electrophoresis of the wild-caught male, while *Fn* heterozygotes are indicated by the presence of S progeny in crosses with the *SS* tester stock. In a similar fashion, F progeny in crosses of S males to the *FF* tester stock distinguishes a *Sn* heterozygote from a *SS* homozygote.

A disadvantage of this method is that large numbers of virgin females from both tester stocks are required and these must be collected prior to, or during field collections.

In this Chapter I describe the frequency of *Adh* and *Gpdh* null alleles detected in the Australian populations over three years. All the *Gpdh* putative null alleles isolated were later shown by Dr J.B. Gibson to be low activity variants but in the descriptions of their frequencies below they are referred to as 'null alleles'. In addition viability data on *Adh* null bearing second chromosomes is presented and discussed in relation to the frequencies.

3.2 Materials and Methods

3.2.1 Collections

The populations sampled and collections made in 1983 have been described in Chapter Two. Collections were again made from these populations in 1984.

At Cygnet and Tamar, population locations and conditions were similar to those found in 1983; flies at the Cygnet apple processing factory were breeding on decomposing apple pulp, while at the Tamar winery flies were again collected from over piles of discarded grape pressings. However, the Huonville (I) site (a waste apple dump in a field adjacent to an apple processing factory) no longer had apple waste

and so a site about 3km from Huonville was located, where apple processing waste had been dumped for cattle fodder (Huonville II, Avondale Farm). The Pipers Brook population, although sampled, was not included in the null allele survey as I preferred to screen a large number of flies from one population (Tamar) rather than low numbers from all the populations. These 1984 Tasmanian collections were surveyed for null alleles by the tester stock method described; males were crossed in turn to females of both 1983 Cygnet tester stocks and their progeny phenotypes analysed.

For comparison with the Tasmanian population data, another population was required which fulfilled several criteria: the population had to be geographically distant to Tasmania and yet should have similar frequencies of the common Adh^F and Adh^S alleles. Also for the comparisons to be meaningful the population should be breeding on a similar resource, i.e., apple processing waste or grape skins. An extensively studied population (Anderson and Gibson, 1985; Gibson, *et al.*, 1981; Gibson and Wilks, 1986) located at the All Saints winery at Rutherglen (Victoria) met these criteria; the site was geographically distant, the Adh^F frequency was about 0.68 (comparable to the average 0.63 found in the 1983 Tasmanian populations) and flies were breeding on discarded grape pressings. In addition, two other breeding resources were available inside the winery; leakages from barrels of fortified wines, and open fermentation vats. These three resources together provided habitats of decreasing ethanol concentrations from inside to outside the winery with mean ethanol levels of 3.16, 2.17, and 1.26 percent (v/v) for barrels, vats, and grape dumps respectively (Gibson and Wilks, 1986). Therefore, in 1984 collections were made near leakages from barrels and over open fermentation vats inside the winery

and at grape skin dumps about 500m from the winery. These All Saints population samples were screened for null alleles by the progeny testing method used in 1983.

In 1984 large samples of flies collected from each of the Tasmanian populations and at Rutherglen were exposed to pentenol vapour within 24 hours of capture. As in 1983 all adults were net swept and transported on standard laboratory media.

In addition, further collections were made by Dr J.B. Gibson at Avondale Farm (Huonville II) and Tamar Valley in May 1985 and analysed by the female progeny method. As well, a collection was made from apple waste dumped for livestock fodder at Nelson, New Zealand, in December 1985 and Dr A. Gibbs provided a sample of flies he collected breeding on bananas and mangoes at Cardwell (Queensland) during October 1985. Both the Nelson and Cardwell collections were screened for null alleles by the 1983 progeny testing procedure. Overall, the collection sites spanned 25 degrees of latitude on a north-south axis (Fig. 3.1).

3.2.2 Viability and lethal chromosome studies

The relative viabilities of flies homozygous for extracted chromosomes carrying *Adh* null activity alleles were estimated from the proportion of wild-type progeny in $CyO/+_1$ x $CyO/+_1$ single pair crosses. *CyO/+* flies were obtained by mating male null allele homozygotes to females of the $T(2;3)\ ap^{Xa}/CyO;TM6$ stock.

The lethal chromosomes *AC41* and *AC60* were tested in combination by 14 single pair crosses of *CyO/AC41* males to *CyO/AC60* females (Set One) with a further 9 pairs of the reciprocal cross (Set Two) on standard laboratory media. In all matings, flies were homozygous for the wild-type third chromosomes. *AC41*, *AC60*, and another lethal chromosome, *AC62*, were also tested in combination with two *Adh* deficiency stocks

Df(2L)64j and *Df(2L)A379*, both provided by Dr M. Ashburner. The deficiency *Df(2L)A379* extends proximally from the no-ocelli (*noc*) locus up to and including the *Adh* locus (Ashburner, Aaron, and Tsubota, 1982) and is small compared to *Df(2L)64j* which is defined cytologically by *Df(2L)34E5-F1; 35C3-D1* (Lindsley and Grell, 1968). For *AC41* and *AC60* between six and ten single pair matings and one mass culture (six pairs) were set up of the type *AC41* or *AC60* females crossed to male *Df(2L)64j* and *Df(2L)A379* flies. *AC62* was tested by two single crosses of male *AC62* flies crossed to *Df(2L)64j* females.

3.2.3 Persistence of null alleles in laboratory cultures

The extent to which *Adh* null alleles identified in 1983 persisted in mixed culture was ascertained by examination of lines in which the original frequencies of *F*, *S*, and *n* alleles could be predicted. These lines had been insurance bottle cultures derived from the single female lines and were established during the isolation of the 1983 null alleles. After four generations under standard laboratory conditions all flies present were screened by pentenol vapour and survivors were isolated and confirmed to lack ADH activity by electrophoresis or the histochemical spot test. Null allele frequencies were then estimated by taking the square root of null homozygote frequencies.

3.3 Results

3.3.1 Null allele frequencies

The number of anomalous cultures and the derived null allele frequencies found in the 1983 Cygnet samples and in those from the other populations sampled in 1983 are given in Table 3.4. The highest null allele frequency was 3.9% at the *Adh* locus in Cygnet, but no *Adh* nulls were detected in Huonville I, 10km to the north of Cygnet. Tamar and

Coffs Harbour had lower *Adh* null frequencies of 0.7% and 0.8% respectively, while Pipers Brook was intermediate at 1.9%. There was no significant heterogeneity in *Adh* null frequencies between the four Tasmanian populations ($X^2_3 = 5.46$, $p > 0.05$) nor between them and Coffs Harbour ($X^2_1 = 0.67$, $p > 0.05$). The frequency of *Gpdh* 'null alleles' was similar to that of *Adh* in the Tasmanian populations sampled ($X^2_1 = 0.05$, $p > 0.05$) and in Coffs Harbour ($X^2_1 = 0.002$, $p > 0.05$) with frequencies ranging from 0.8 up to 2.7%. Overall, the average frequencies for *Adh* and *Gpdh* 'null alleles' were 1.3% and 1.6% respectively.

Frequencies of null alleles at the *Adh* and *Gpdh* loci for the Tasmanian populations sampled in 1984 are given in Table 3.5. In comparison with the previous year, the frequency of *Adh* null alleles in Cygnet was lower ($X^2_1 = 4.58$, $p < 0.05$) but Tamar was similar for each year ($X^2_1 = 0.07$, $p > 0.05$) while Adh^F frequencies remained the same (X^2_1 = 0.65 and 0.16, $p > 0.05$). The frequency at Huonville II was found to be 3.2%, even though only 94 alleles were tested, but overall there was no significant heterogeneity between the three Tasmanian populations (X^2_2 = 4.93, $p > 0.05$). *Gpdh* 'nulls' were again present at similar frequencies to those in the 1983 Cygnet ($X^2_1 = 0.39$, $p > 0.05$) and Tamar ($X^2_1 = 0.07$, $p > 0.05$) populations with frequencies ranging from 0.2 to 1.3%. $Gpdh^F$ frequencies were not significantly different between years (X^2_1 = 0.59 and 0.06, $p > 0.05$).

In 1984 collections were also made from three locations which differed in ethanol concentrations at the All Saints winery near Rutherglen (Vic.) (Table 3.6). *Gpdh* 'null alleles' were found at all three locations at frequencies ranging from 0.6 to 1.4%. A single *Adh* null was detected in the grape skin dump sample but none were found in the samples from the fermentation vats or barrel leakages. There was,

however, no significant heterogeneity between habitats for *Adh* null frequency (X^2_2 = 1.86, $p > 0.05$) or for *Gpdh* (X^2_2 = 1.86, $p > 0.05$) 'null alleles' which were found at all three habitats. There was significant heterogeneity between the three habitats for Adh^F frequencies (X^2_2 = 7.06, $p < 0.05$), but not for $Gpdh^F$ frequencies (X^2_2 = 2.50, $p > 0.05$). There was no significant difference between habitats inside (barrel and vats) and outside (skin dump) the winery (X^2_1 values, $p > 0.05$) in either *Adh* or *Gpdh* null allele frequencies. Overall, there was a significant difference in null allele frequencies at the *Adh* and *Gpdh* loci (X^2_1 = 6.12, $p < 0.05$).

The 1983 data had suggested that in some southern Tasmanian populations *Adh* null alleles might be at frequencies as high as 4% so it was worthwhile attempting to test whether homozygotes for null alleles were present. Flies were net collected in 1984 from the three Tasmanian populations and exposed to pentenol vapour within 24 hours of capture. At Cygnet, Huonville II, and Tamar an estimated (by weight) 675, 5234, and 1194 *D. melanogaster* adult flies were pentenol screened. None of the flies survived. Given the null allele frequency found at Huonville II in 1984, five null homozygotes out of the 5234 tested (2.5g of adult flies) were expected on Hardy-Weinberg predictions (X^2_1 = 3.20, $p > 0.05$). A similar survey at the Rutherglen All Saints winery failed to detect any homozygotes from 3699 flies tested (barrel, 284; vats, 2017; grape skin dump, 1398) but this population had an *Adh* null frequency of only 0.08%.

In 1985 *Adh* null alleles were not found at Tamar, although the frequency of the Adh^F allele was not significantly different to the frequency found in 1984 (X^2_1 = 0.003, $p > 0.05$). The *Adh* null allele frequency found at Huonville II in 1985 had decreased (Table 3.7), but

was not significantly different to that found in the previous year (X^2_1 = 0.14, $p > 0.05$). Adh^F frequencies at Cardwell and Nelson, New Zealand were consistent with the reported clinal variation in *Adh* allele frequencies (Oakeshott *et al.*, 1982), however, no *Adh* null alleles were detected in the samples from either population (Table 3.7).

Nine *Adh* null alleles were isolated from the 1983 material as described in Chapter Two (seven from Cygnet, one from Tamar and one from Pipers Brook) while seven chromosomes bearing *Adh* null alleles were isolated with Dr J.B. Gibson and Ms A.V. Wilks from the 1984 samples using the second chromosome balancer *CyO* or by pentenol screening. All of the *Adh* nulls lacked ADH activity on cellulose acetate membranes either as homozygotes or when heterozygous with an *Adh* deficiency. Apart from three Cygnet nulls isolated in 1983 and one from Huonville II in 1984, which were homozygous lethal, all survived 4 minutes exposure to pentenol vapour as homozygotes.

Five putative *Gpdh* null alleles were isolated by Dr J.B. Gibson and Ms A. Cao from the 1984 Cygnet, Tamar, and Huonville collections by the *CyO* breeding programme and all were found to be low activity variants. Likewise, in 1985 Dr J.B. Gibson and Ms A. Cao isolated the seven *Adh* null alleles detected in the 1985 Huonville II (Avondale Farm) collections.

To identify null activity alleles I have followed the protocol of Burkhart *et al.* (1984) by specifying the population and line from which the allele was extracted. Thus Adh^{nAC14} designates a null extracted from the 14th single female line of the Australian Cygnet population.

3.3.2 Chromosome viabilities

Of the 16 chromosomes isolated in 1983 and 1984 bearing *Adh* null alleles 12 were homozygous viable and four were lethal as homozygotes. Two, from Huonville II, were homozygous viable but the males and females were sterile (*AH41* and *AH108*). All 16 were found to be viable and fertile as heterozygotes with the *Adh* deficiency *Df(2L)64j* (Table 3.8 lists data for the six 1983 homozygous viable chromosomes).

3.3.2.1 Homozygous viable chromosomes

Table 3.9 lists all sixteen chromosomes and the relative viabilities of the eight tested together with data for six other second chromosomes bearing active *Adh* alleles extracted at the same time. Of the eight homozygous viable null bearing chromosomes four (*AC22*, *AC80*, *AC95* and *AH41*) were significantly less viable as homozygotes than when heterozygous with *CyO*. None of the six chromosomes with active *Adh* alleles were lethal, but three of them (*AC28*, *AC34*, and *ATC1*) had lower viabilities as homozygotes than when heterozygous with *CyO*. Both 1983 second chromosomes from Cygnet carrying null alleles had higher relative viabilities than their respective control chromosomes bearing active *Adh* alleles.

3.3.2.2 Homozygous lethal chromosomes

Three second chromosomes from the 1983 Cygnet collection, *AC41*, *AC60*, and *AC62* were found to be lethal when homozygous and were maintained in a balanced lethal stock with *CyO* and *TM6*. The lethality associated with *AH36* from the 1984 Huonville II collections was separable from the *Adh* locus by recombination and this null allele can now be maintained as a homozygote.

AC41 and *AC60* were tested in combination by reciprocal matings. As no difference was found between the progenies of the reciprocal matings

(X^2_1 = 0.47, $p > 0.10$) all the data was pooled. Of the 527 progeny scored, all were found to be *CyO*; the lack of wild-type progeny indicating these two chromosomes carried the same lethal.

To test whether this lethality was located at or near the *Adh* locus the three lethal chromosomes were made heterozygous with the deficiencies *Df(2L)64j* and *Df(2L)A379*.

Progeny data from the single pair matings and the mass cultures were combined, as in only one case was significant heterogeneity detected (X^2_1 = 5.23, $p < 0.05$). This was for the *AC60* x *Df(2L)64j* cross and was believed to be due to the relatively low number of F1 progeny produced by the single pair matings.

As both the lethal and deficiency chromosomes were balanced by *CyO* the expected ratio of *CyO* to wild-type progeny is 2:1 assuming that the lethal/deficiency heterozygotes are viable. The numbers of *CyO* and wild-type progeny are shown in Table 3.10. From all crosses wild-type progeny were recovered and confirmed to lack ADH activity after electrophoresis. Phenotype ratios of *CyO* to wild-type progeny were as expected for *Df(2L)64j* matings assuming lethal/deficiency heterozygotes were viable. However, in both crosses with *Df(2L)A379* fewer *CyO* individuals than expected were recovered, reflecting the reduced viability of *CyO/Df(2L)A379* individuals (Dr J.B. Gibson, *personal communication*).

3.3.3 Persistence of null alleles

In Table 3.11 the *Adh* null frequencies detected in various lines after four generations of laboratory culture are listed. Overall, there was a general trend for null allele frequencies to decline with 10 out of the 18 lines screened showing reductions. Three lines *AC22* (*SS*, *Sn*, *Fn*, *FS*), *AC53* (*FF*, *Fn*, *nn*), and *AC53* (*Fn*, *Sn*, *FS*, *nn*) showed little

change while in one, *AC53* (*FF*, *Fn*, *Sn*, *FS*), the null frequency nearly doubled. In the lines containing the allele *AC60* no *Adh* null homozygotes were detected. Their second chromosomes originally were homozygous lethal. Of the three *AC62* lines which had also been shown to be homozygous lethal one did produce some null allele homozygotes.

3.4 Discussion

The average frequency of *Adh* null alleles in the 1983 collections from four Tasmanian populations was 1.6%. Inclusion of the Coffs Harbour data reduced this average to 1.3% which remains fourteen times the value reported by Langley *et al.* (1981) for their Raleigh and London samples. Overall, *Adh* null allele frequencies were lower in 1984 than in the previous year, but the average null frequency in the Tasmanian populations remained over seven times higher than reported by Langley *et al.* (1981) who detected only one *Adh* null allele out of 1170 alleles screened (0.09%). On further investigation this was found to be a low activity allele (Dr M. Ashburner, *personal communication*). *Adh* null frequencies in other Australian populations seemed to be lower than in Tasmania, but with such low frequencies sampling variances will affect comparisons and more extensive sampling will be required to fully assess the overall frequencies and to compare them with those found by Langley *et al.* (1981).

Interpretation of the differences in *Adh* null allele frequencies between the 1983 and 1984 Tasmanian population samples is confounded by the different methods used to detect null alleles in the two years. The Adh^F frequency was constant between years and although there are too few data for a critical test they suggest that sampling variance could explain the observed heterogeneity in the null frequency between years, and between populations, given the *Adh* null allele frequencies. The

Tasmanian populations suffer a dramatic bottleneck after May and do not appear to breed over winter. But breeding occurs all year round at Coffs Harbour even though winter population sizes are greatly reduced.

The 1983 progeny testing method was thought to underestimate the true null allele frequency, but by less than 1% (Cygnet 4.8%, Tamar 1.0%, Pipers Brook, 2.3% and Coffs Harbour 0.9%). The accuracy of the correction (see Section 3.1) is dependent on the population being in, or near to, Hardy-Weinberg equilibrium. In any case, with the low frequency of nulls sampling variance is likely to have a greater effect on frequency estimates than such a bias.

The absence of *Adh* null alleles inside the All Saints winery is intriguing, as it parallels both the levels of ethanol detected in the breeding sites and levels of ADH activity which are higher inside than outside the winery (Gibson and Wilks, 1986). *Adh* null heterozygotes have about half the ADH activity of normal homozygotes (Chapter Four); so that Adh^S/null heterozygotes have similar levels of ADH activity to *D. simulans* which is rare inside the winery. Nevertheless, the role of ADH activity in ethanol tolerance and metabolism remains unclear (Kerver and van Delden, 1985; Middleton and Kacser, 1983) even though homozygotes for artificially induced *Adh* null alleles die when exposed to 6% ethanol (Vigue and Sofer, 1976). The ethanol tolerance of homozygotes for *Adh* null alleles isolated from the Tasmanian populations has been investigated and is discussed in relation to ADH activity in Chapter Four.

The data for the putative *Gpdh* null alleles are difficult to interpret because subsequent investigation of the alleles isolated in 1984, and in 1985 by Dr J.B. Gibson, revealed that they all had low levels of GPDH activity. The initial identification of these putative

null alleles had been based on their allozyme patterns when heterozygous with *F* or *S* alleles. Following the criteria of Voelker *et al.* (1980a) a mutant allele was scored as a null if it failed to produce a mutant homodimer regardless of the level of residual activity in the heterodimer. Whereas all the *Adh* null alleles detected showed no allozyme activity as either homodimers or heterodimers, residual heterodimer activity was often observed for the *Gpdh* nulls during the screening programme. Langley *et al.* (1981) had identified ten putative *Gpdh* null alleles out of 1200 tested from the Raleigh and London samples. Seven of these were later found to be low activity variants (Burkhart *et al.*, 1984) giving an average frequency of 0.58% for the two populations. This is comparable to the average frequency of *Gpdh* low activity variants (0.5%) found in the 1984 Tasmanian populations, although the 1983 collections from the Tasmanian populations had a much higher frequency of *Gpdh* variation (1.8%). There is no evidence that the active *Gpdh* alleles differed significantly between years. The relative proportions of null and low activity variants in the 1983 samples are unknown as only one allele was isolated and this had low GPDH activity. Results from experiments carried out in 1985 by Dr J.B. Gibson in the Huonville II population provided further evidence on this point. All the aberrant *Gpdh* alleles detected proved on analysis to be low activity variants. In contrast the data from the same populations for *Adh* shows that all aberrant alleles totally lack ADH activity.

The frequency of *Adh* null alleles found in the Tasmanian populations is unexpectedly high compared to the data of Langley *et al.* (1981). Any attempts to explain these frequencies must take into account the number of different null alleles, mechanisms which might enhance mutation rates, and the possibility of selective maintenance of null alleles in heterozygotes.

The viability tests show that amongst 12 *Adh* null bearing chromosomes, four initially carried recessive lethals, a further four were of lower viability as homozygotes than when heterozygous with *CyO*, and two were sterile as homozygotes. The Cygnet population which provides the largest sample of *Adh* null bearing second chromosomes for comparison shows some evidence for intra-population heterogeneity. Of eight Cygnet second chromosomes carrying *Adh* null alleles three are homozygous lethal and two of these are lethal in combination. Three other Cygnet second chromosomes have significantly reduced viability and a further two, although viable as homozygotes, were not tested in comparison with *CyO*. If all the Cygnet *Adh* null alleles are indeed descendants of a single mutational event, then they have remained in the population long enough for the second chromosomes to become differentiated with respect to their viabilities. Cytology was not useful in discriminating between second chromosomes as the average frequency of second chromosome inversions in the Tasmanian populations is very low, less than 0.2 inversions per fly (Knibb *et al.*, 1981). Dr I. Boussy (*personal communication*) confirmed this observation in a study of the May 1983 Tasmanian samples collected by Dr J.B. Gibson and Ms A.V. Wilks. Resolution of how many different null alleles are present requires molecular and biochemical analyses (Chapters Five, Six, and Seven).

Overall, the distribution of viabilities in second chromosomes containing *Adh* null alleles is not dissimilar to that found in earlier surveys of 100 second chromosomes from each of the Cygnet and Tamar populations, in which an average of 32% homozygous lethals were detected (Gibson, unpublished results). It is worth noting that chromosomes bearing *Adh* null alleles were often more viable as homozygotes than

those with active alleles when compared to their respective *CyO* heterozygotes.

In natural populations the *Adh* null alleles are maintained as heterozygotes with normal alleles. Langley *et al.* (1981) have calculated the selective disadvantage of null heterozygotes to be 0.0015 by the equation $q = u/hs$ where q is the frequency of a null allele, u is the mutation rate to null alleles (3.86×10^{-6}, Voelker *et al.*, 1980b), h is the measure of dominance, and s is the reduction in fitness of the null homozygote. The average frequency of *Adh* nulls that we observed in the 1983 sample gave a figure of 0.0003. Although the hs term cannot be further resolved, it appears that these nulls have little deleterious effect in heterozygotes with normal alleles.

The laboratory lines did show a marked decrease of null allele frequency in mixed allele cultures, but this may be the result of crowded laboratory culture conditions. The lines were not monitored beyond four generations to see if the null allele was eliminated.

In the population samples the *Adh* null alleles were not found as heterozygotes with predominantly one active allele. In 1983 nine Adh^n/Adh^F and four Adh^n/Adh^S heterozygotes were found while in 1984 the number was three and four respectively. This is an interesting observation in view of the greater activity of ADH-F compared to ADH-S (Gibson, 1972; Day, Hillier, and Clarke, 1974).

It is possible that some mechanism in the Tasmanian populations might increase the mutation rate to null alleles at the *Adh* locus. Assuming that null allele frequencies are determined solely by mutation-selection balance then the mutation rate to null alleles can be estimated using the equation $q = \sqrt{u/s}$, where q is the null allele frequency, u the mutation rate, and s the selection coefficient against

null homozygotes. The average frequency of *Adh* nulls in 1983 was 0.013, which would require a mutation rate of 10^{-3} if s was 1.0. This is considerably higher than the spontaneous mutation rate to null alleles of 3.86×10^{-6} reported by Voelker *et al.* (1980b). In fact, they did not detect any spontaneous *Adh* null mutants, although an earlier screen of the same lines (Mukai and Cockerham, 1977) did detect one *Adh* null. In that study, Mukai and Cockerham (1977) estimated a higher null mutation rate of 1.03×10^{-5}, which is still much lower than the estimate based on the 1983 data. Mutation rates as high as 10^{-4} to 10^{-3} have been reported for the X-linked singed bristle locus of *D. melanogaster* (Golubovsky, Ivanov, and Green, 1977) and been attributed to male recombination (Green, 1977).

Male recombination is one of a variety of traits associated with hybrid dysgenesis. Other effects include mutation, sterility, segregation and sex ratio distortion, and chromosomal aberrations (Kidwell *et al.*, 1977; Engels, 1981). These traits occur in hybrid progeny produced from a mating between P strain males (predominantly recent wild strains) and M strain females (predominantly laboratory strains). There is some indirect evidence that P-factors are unlikely to be the cause of the relatively high frequency of *Adh* null alleles in the Tasmanian populations.

Dr I. Boussy (*personal communication*) in a test for potentially active P-elements has found little gonadal dysgenesis (3%) in the F1 between male flies from the Cygnet population and a standard M strain, while in a similar test Coffs Harbour flies produced a mean F1 gonadal dysgenesis of 35%. Also, flies from another site (Chateau Lorraine), about five kilometres from Cygnet gave a mean percentage of dysgenic F1 females of less than one. In the reciprocal test of cytotype

susceptibility to P activity where female Cygnet flies were crossed to a standard P strain, 47% of F1 females had one or both ovaries undeveloped whereas only 3% of similar Coffs Harbour F1 females were dysgenic. Consequently, enhanced null mutation rates due to P-element activity in the Tasmanian populations were considered unlikely, although mutations due to other groups of mobile elements cannot be ruled out without molecular studies.

In addition, a survey of 30 second chromosomes found no male recombination in flies from Cygnet or Tamar, but male recombination does exist in the Coffs Harbour population (Dr M. Green and Dr J.B. Gibson, *personal communication*). Also, the frequency of second chromosome recessive lethals does not differ significantly between the Tasmanian and Coffs Harbour populations (Gibson, unpublished results). So far, there is little evidence of genetic mutator factors acting to increase mutation rates in the Tasmanian populations, but the possibility remains.

Woodruff, Thompson, Seeger, and Spivey (1984) have reported considerable variation in spontaneous mutation rates and repair ability in population lines of *D. melanogaster* from an Australian winery. When the population was small (i.e., before the expansion during the grape harvest), mutation rates were found to be higher than in high density populations present after harvest. This may be of importance to the Tasmanian populations which are known to suffer winter bottlenecks (extending from June to October) before expanding to large populations in late summer and autumn.

To conclude, the frequencies of *Adh* null alleles found in the Tasmanian populations contrast with those obtained elsewhere in Australia and with those in previous surveys in the Northern

Hemisphere. At least in some of the Tasmanian populations *Adh* null alleles are maintained at, or close to, polymorphic frequencies. It remains possible that high mutation rates might be responsible for the high frequencies of nulls and the molecular analysis of these alleles will be required to elucidate this possibility. Such analyses, together with biochemical studies, will facilitate the detection of heterogeneity amongst the *Adh* null alleles and help to resolve the questions concerning their origin.

Table 3.1. Phenotype classes produced by the six genotypes in the 36 possible mating combinations (n = no activity).

Female genotype	Male genotype					
	FF	*Fn*	*SS*	*Sn*	*FS*	*nn*
FF	F	F	FS	F.FS	F.FS	F
Fn	F	F.n	S.FS	F.S.FS.n	F.S.FS	F.n
SS	FS	S.FS	S	S	S.FS	S
Sn	F.FS	F.S.FS.n	S	S.n	F.S.FS	S.n
FS	F.FS	F.S.FS	S.FS	F.S.FS	F.S.FS	F.S
nn	F	F.n	S	S.n	F.S	n

Table 3.2. Expected phenotype classes for the progeny of females apparently homozygous (F or S) classified according to whether a null allele is present or undetectable.

Electrophoretic phenotype of female parent	Progeny classes with electrophoretic phenotypes		
	Null allele present	Null allele absent	Null allele undetectable
F (*FF* or *Fn*)	(i) S and FS (ii) F, S, and FS (iii) F, S, FS, and n (iv) F and n	(i) all FS	(i) all F
S (*SS* or *Sn*)	(i) F and FS (ii) F, S, and FS (iii) F, S, FS, and n (iv) S and n	(i) all FS	(i) all S

Table 3.3. Progeny phenotypes expected from the six possible male genotypes crossed to females of the Cygnet *FF* and *SS* tester stocks.

Genotype of male	Phenotype of male	Progeny phenotypes from crosses to the *FF* tester stock	Progeny phenotypes from crosses to the *SS* tester stock
FF	F	F	FS
Fn	F	F	S FS
SS	S	FS	S
Sn	S	F FS	S
FS	FS	F FS	S FS
nn	n	F	S

Table 3.4. Null allele frequencies at the *Adh* and *Gpdh* loci for the populations sampled in 1983.

Population sampled	Lat. °South	Long. °East	*Adh*					*Gpdh*				
			Single female lines	Homozygous lines	Lines with evidence of null allele	Null allele frequency %	*F* allele frequency %	Single female lines	Homozygous lines	Lines with evidence of null allele	Null allele frequency %	*F* allele frequency %
Cygnet (TAS.)	43.1	147.0	78	44	6	3.9	58.3	75	38	4	2.7	70.6
Huonville I (TAS.)	43.0	147.0	79	36	0	0	57.6	N.T.	-	-	-	-
Tamar (TAS.)	41.2	146.5	69	43	1	0.7	73.2	66	34	1	0.8	65.9
Pipers Brook (TAS.)	41.1	147.1	81	41	3	1.9	62.3	N.T.	-	-	-	-
Coffs Harbour (N.S.W.)	30.2	153.1	187	84	3	0.8	42.5	84	50	2	1.2	72.6
Average null allele frequencies						1.3					1.6	

N.T. - not tested.

Table 3.5. Null allele frequencies at the *Adh* and *Gpdh* loci for the populations sampled in 1984.

Population sampled	*Adh*			*Gpdh*		
	Males tested	Null allele frequency %	*F* allele frequency %	Males tested	Null allele frequency %	*F* allele frequency %
Cygnet (TAS.)	120	0.4	62.9	119	1.3	66.4
Huonville II (TAS.)	47	3.2	53.2	43	1.2	63.9
Tamar (TAS.)	323	0.5	71.0	304	0.2	67.4
Average null allele frequency		0.7			0.5	

Table 3.6. Frequencies of null alleles at the *Adh* and *Gpdh* loci for the three locations sampled at the All Saints Winery, Rutherglen, (36° 0', 146° 3').

Habitat sampled	*Adh*					*Gpdh*				
	Single female lines	Homozygous lines	Lines with evidence of null allele	Null allele frequency %	*F* allele frequency %	Single female lines	Homozygous lines	Lines with evidence of null allele	Null allele frequency %	*F* allele frequency %
Leakages from barrel of fortified wine	185	113	0	0	65.4	161	97	2	0.6	74.8
Fermentation vats	211	107	0	0	62.1	182	101	2	0.6	69.5
Grape skin dump	213	130	1	0.2	70.7	178	90	5	1.4	71.1

Table 3.7. *Adh* null allele frequencies for three Australian and one New Zealand population sampled in 1985.

Population sampled	Latitude South°	Longitude East°	Single female lines	Homozygous lines	Lines with evidence of a null allele	Null allele frequency %	*F* allele frequency %
Huonville II (TAS.)	43.0	147.0	185	99	7	1.9	57.3
Tamar (TAS.)	41.2	146.5	102	64	0	0	71.6
Cardwell (QLD.)	18.2	146.0	49	39	0	0	10.2
Nelson (N.Z.)	41.2	173.2	119	60	0	0	65.1

Table 3.8. Progeny from crosses between the 1983 null allele homozygotes and flies heterozygous for the *Adh* deficiency *Df(2L)64j*. Numbers represent combined female and male results pooled from two separate crosses of single male nulls to three *CyO/Df(2L)64j* females. X^2 goodness of fit tests for deviations from a 1:1 ratio of *CyO* to wild-type individuals.

Cross	Progeny phenotypes		X_1^2
	CyO	Wild-type	
AC14 x *CyO/Df(2L)64j*	115	97	1.53
AC22 x *CyO/Df(2L)64j*	73	77	0.06
AC53 x *CyO/Df(2L)64j*	81	67	1.14
AC80 x *CyO/Df(2L)64j*	92	54	9.38
AP81 x *CyO/Df(2L)64j*	77	76	0
AT33 x *CyO/Df(2L)64j*	72	62	0.60

Table 3.9. Relative viabilities, compared with *CyO*, of the *Adh* null bearing second chromosomes extracted from the Tasmanian populations. Also included are chromosomes with active alleles (†) extracted at the same time.

Year sampled	Population	Chromosome	Relative viability
1983	Cygnet	*AC14*	N.T.
1983	Cygnet	*AC22*	0.81***
1983	Cygnet	*AC41*	Lethal
1983	Cygnet	*AC53*	N.T.
1983	Cygnet	*AC60*	Lethal
1983	Cygnet	*AC62*	Lethal
1983	Cygnet	*AC80*	0.69***
1983	Cygnet	*AC28*†	0.47***
1983	Cygnet	*AC34*†	0.68**
1983	Tamar	*AT33*	N.T.
1983	Pipers Brook	*AP81*	N.T.
1984	Cygnet	*AC95*	0.46***
1984	Huonville II	*AH36*	Lethal
1984	Huonville II	*AH41*	0.55***
1984	Huonville II	*AH108*	1.03
1984	Huonville II	*AHC1*†	0.88
1984	Huonville II	*AHC2*†	0.94
1984	Tamar	*AT240*	1.05
1984	Tamar	*AT265*	0.82
1984	Tamar	*AT340*	1.01
1984	Tamar	*ATC1*†	0.65***
1984	Tamar	*ATC2*†	1.02

** $p < 0.01$, *** $p < 0.001$.

Table 3.10. Progeny from crosses between the lethal null bearing chromosomes *AC41*, *AC60*, *AC62*, and chromosomes bearing the *Adh* deficiencies *Df(2L)64j* and *Df(2L)A379*. X^2 goodness of fit tests for deviations from a 2:1 ratio.

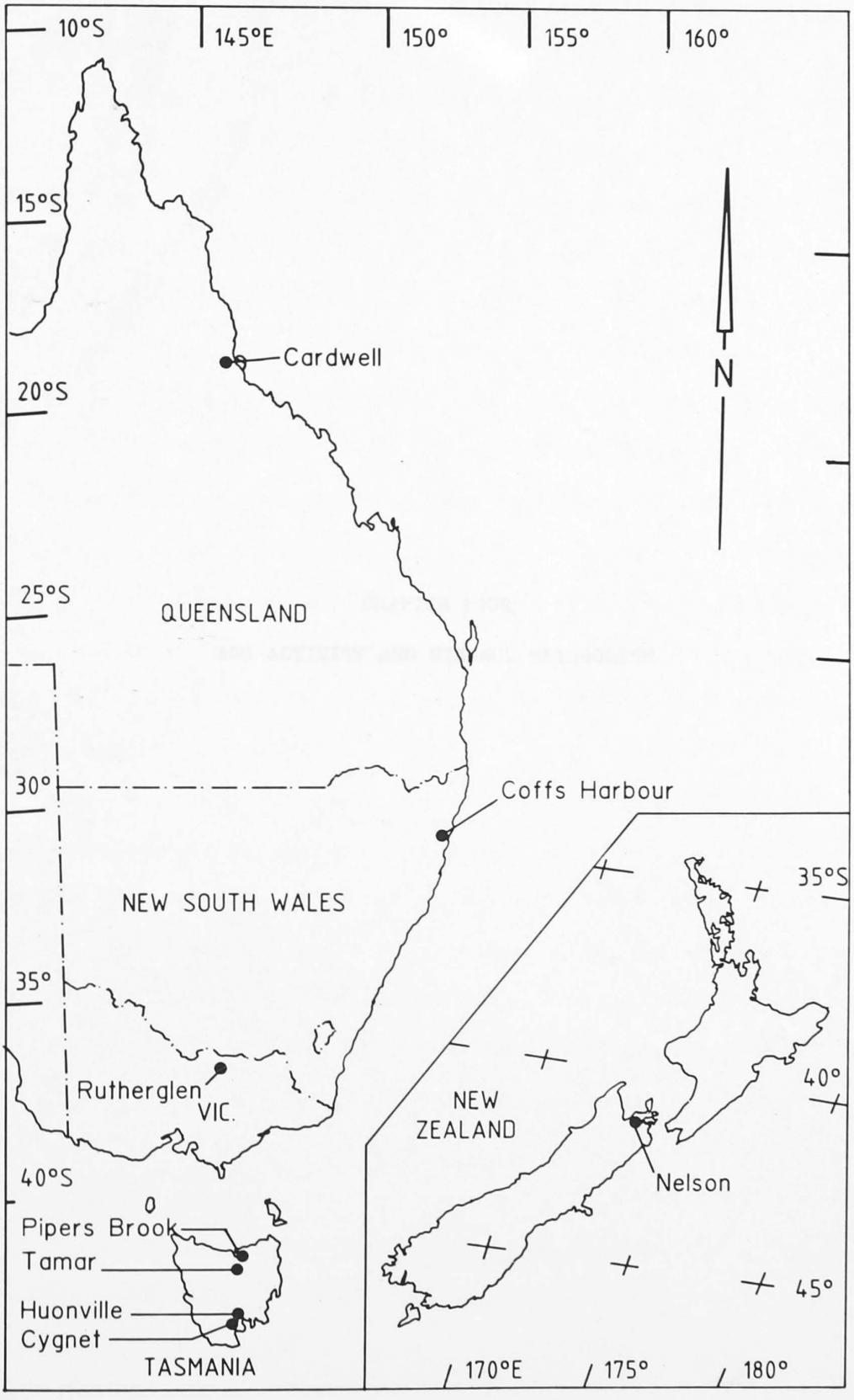
Cross	Progeny phenotypes		
	CyO	Wild-type	X^2_1
CyO/AC41 x *CyO/Df(2L)64j*	182	73	2.54
CyO/AC60 x *CyO/Df(2L)64j*	64	34	0.08
CyO/AC62 x *CyO/Df(2L)64j*	47	23	0
CyO/AC41 x *CyO/Df(2L)A379*	111	136	52.47***
CyO/AC60 x *CyO/Df(2L)A379*	60	60	15.00***

***$p < 0.001$

Table 3.11. Null allele frequencies after four generations in lines derived from different single females.

Presumed genotypes in original line	Null allele	Original null allele frequency	Number of flies screened	Survivors recovered	Estimated null allele frequency
FF Fn Sn FS	*AC53*	0.25	275	60	0.47
	AP81	0.25	279	1	0.06
Fn Fn FS FS	*AC14*	0.25	315	3	0.10
	AC62	0.25	445	0	0
SS Sn Fn FS	*AC14*	0.25	371	6	0.13
	AC22	0.25	633	49	0.28
	AC60	0.25	512	0	0
	AC62	0.25	209	0	0
	AT33	0.25	143	3	0.14
FF Fn Fn nn	*AC53*	0.50	227	55	0.49
	AP81	0.50	94	12	0.36
Fn Sn FS nn	*AC14*	0.50	365	18	0.22
	AC53	0.50	357	64	0.42
	AC62	0.50	198	4	0.14
	AT33	0.50	136	1	0.09
SS Sn Sn nn	*AC14*	0.50	211	14	0.26
	AC22	0.50	391	39	0.32
	AC60	0.50	383	0	0

Figure 3.1. Location of the Australian and New Zealand *D. melanogaster* populations surveyed for *Adh* null alleles.


10°S
145°E
150°
155°
160°
15°S
20°S
25°S
30°
35°
40°S
N
Cardwell
QUEENSLAND
Coffs Harbour
NEW SOUTH WALES
Rutherglen
VIC.
Pipers Brook
Tamar
Huonville
Cygnet
TASMANIA
NEW
ZEALAND
Nelson
35°S
40°
45°
170°E
175°
180°

CHAPTER FOUR

ADH ACTIVITY AND ETHANOL METABOLISM

4.1 Introduction

Any attempt to explain the relatively frequent occurrence of null alleles in the Tasmanian populations must take into account the number of different null alleles and consider the mechanisms likely to be responsible for their maintenance in natural populations. Each of the null alleles maintained was separately extracted from a wild-caught fly but as yet there is no evidence that they represent different mutations.

As a first step in addressing these questions it is important to determine if any of the putative *Adh* null alleles show residual activity as homozygotes. The level of ADH activity and ADH protein in null/active allele heterozygotes is also important, as these null alleles will be present as heterozygotes in the Tasmanian populations. Investigation of these properties may indicate heterogeneity among the separately extracted null alleles and provide information pertaining to their maintenance in natural populations.

Since the original observation by Grell *et al.* (1968) that ADH negative flies die in the presence of ethanol, the biological role of ADH has been considered to be the detoxification of environmental ethanol. Later studies have shown that ADH can contribute to the energy metabolism of the fly (Libion-Mannaert, Delcour, Deltombe-Lietaert, Lenelle-Montfort, and Elens, 1976; van Herrewege and David, 1980; Daly and Clarke, 1981). Ethanol *in vivo* is believed to be transformed by ADH into acetaldehyde which is subsequently converted to acetate, then to acetyl-CoA for metabolic use (Heinstra *et al.*, 1983). However, the association of ADH activity with tolerance to environmental ethanol in *D. melanogaster* is a matter of some controversy.

Although earlier studies on inbred material found a correlation in laboratory experiments between the frequency of the Adh^F allele, ADH activity, and ethanol tolerance (Gibson, 1970; Bijlsma-Meeles and van Delden, 1974; van Delden, Boerema, and Kamping, 1978), later studies on a series of Australian winery populations and on outbred material demonstrated that ethanol tolerance could be independent of the ADH system (McKenzie and Parsons, 1974; McKenzie and McKechnie, 1978; Gibson, Lewis, Adena and Wilson, 1979; Gibson *et al.*, 1981; Oakeshott *et al.*, 1983). Nevertheless, the discovery of *Adh* null alleles at relatively high frequencies in natural populations is surprising and emphasises the uncertainty about the role of ADH in ethanol tolerance.

This chapter describes the partial biochemical characterisation of the *Adh* null alleles isolated in 1983, both as homozygotes and null/active allele heterozygotes, together with ethanol tolerance experiments using *Adh* nulls isolated in 1983 and 1984.

4.2 Materials and Methods

4.2.1 *Drosophila* strains

Several *D. melanogaster* strains, apart from the *Adh* null alleles isolated in 1983 and 1984, were used in these experiments. Three EMS-induced null mutants, Adh^{n7}, Adh^{n10}, and Adh^{n11} described by Schwartz and Sofer (1976) were provided by Dr M. Ashburner and Professor W. Sofer. In addition, two strains homozygous for Adh^F and Adh^S alleles, designated *AC5* and *AC8* respectively, were used as controls. These alleles were isolated from the Cygnet population sampled in 1983 and had been used as the two tester stocks in the 1984 Tasmanian null allele survey. In addition, lines homozygous for an Adh^S and also an Adh^F allele isolated by Lewis and Gibson (1978) were used in the experiments assaying ADH activity and protein.

4.2.2 ADH activity and complementation experiments

All crosses were made with 20 pairs of male and female flies in 250 ml bottle cultures at 25°C. The four 1983 null alleles, Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, and Adh^{nAC80} were crossed to themselves and each other in a half-diallel scheme with two mass crosses for each combination. Also, Adh^{nAC22} and the remaining two 1983 homozygous viable alleles, Adh^{nAP81} and Adh^{nAT33}, were crossed in a similar scheme, but with only one mass cross for each possible combination. Cultures of an Adh^{S} allele (Lewis and Gibson, 1978) provided controls. Third instar larvae from the first set of crosses were collected and classified by electrophoresis for ADH genotypes, after which two collections of adult male progeny from all crosses were used in extracts to determine ADH activity spectrophotometrically (Section 4.2.4). For all assays in which low levels of ADH activity were expected the extract assay volumes were increased to 200ul and the Gilford spectrophotometer set at the most sensitive scale.

In a separate experiment, ADH activity was assayed in 3rd instar larval extracts from four cultures (initiated by 20 pairs of parents) of Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAC80}, Adh^{nAP81}, Adh^{nAT33}, and a control Adh^{S} allele.

During both experiments serial dilutions of the Adh^{S} extracts were assayed to determine the sensitivity of the assay. Also, background endogenous NAD reduction was monitored in Adh^{S} extracts in the presence of 0.1M pyrazole which inhibits ADH activity (Borack and Sofer, 1971).

Complementation between an EMS-induced null, Adh^{n11} and the six 1983 homozygous viable alleles was tested for by the presence of an active heterodimer in null/Adh^{n11} heterozygotes. Mass crosses of natural null males to Adh^{n11} females were set up together with a control

cross of Adh^{n7} males to Adh^{n11} females, as Adh^{n7}/Adh^{n11} heterozygotes were reported to show complementation (Schwartz and Sofer, 1976). Heterozygous progeny from these crosses were classified after electrophoresis for the presence of a heterodimer of residual activity.

4.2.3 Production of heterozygotes for assays

All crosses were of 20 pairs of flies on standard laboratory media in 250ml bottles. Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAC80}, and Adh^{n10} were mated reciprocally to standard Adh^{F} and Adh^{S} strains (Lewis and Gibson, 1978). For Adh^{nAP81}, Adh^{nAT33}, and Adh^{n11} only one cross (male nulls x active allele females) was set up for each null. From all of these cultures one to six separate collections of adult progeny (aged six to seven days) were made, from which between two and twelve extracts of male flies were made for spectrophotometric and immunological analyses.

Levels of *Adh* protein were measured in all extracts assayed for ADH activity. The remaining F1 progeny, both male and female, were tested for the presence of an active heterodimer by electrophoresis as previously described in Chapter Two.

4.2.4 ADH activity assays

ADH activity assay conditions were slightly modified from those used by Gibson *et al.* (1980). Adult male flies, aged for six to seven days, were anaesthetised by ether, weighed and then homogenised in ground-glass tissue grinders in ice cold 100mM sodium phosphate buffer (pH7.5) to a final concentration of 10mg/ml live weight. Third instar larvae homozygous for some null alleles were also assayed. The homogenates were centrifuged at 10,000rpm (Sorvall SM-24 rotor) for 30 minutes after which supernatants were stored on ice until assayed. Two replicate samples of each extract were assayed. ADH activity was

measured in a 1ml reaction mixture consisting of 150mM isopropanol and 2mM NAD in 100mM sodium phosphate buffer (pH7.5) with between 10 to 200 ul of extract depending on the genotype assayed. The reaction was monitored at 340nm to record NADH production for 2 to 3 minutes with a Gilford 250 spectrophotometer. One unit of activity is defined as an increase in absorbance at 340nm of 0.001/minute at 25°C (i.e., 1.61 x 10^{-4}umoles NADH produced/minute). Enzyme activities are expressed as units per milligram live weight and are termed activity units.

4.2.5 ADH antisera production

ADH antisera was obtained by injecting rabbits with crude extracts of flies homozygous for the ADH-F allozyme. Homogenates were made by grinding 50 ADH-F *D. melanogaster* in 1ml of water. Samples (10 ul) of these homogenates were loaded onto 7% acrylamide gels (5mm diameter x 70mm) made with water and polymerised by 0.1% ammonium persulphate and 0.03% tetramethylethylenediamine (TEMED). After electrophoresis (1 to 2 hours at 60mA with a Tris glycine buffer, pH 8.5) the gels were stained for ADH as described in Chapter 2. Staining was stopped as soon as the ADH-F band was visible and the central region of this band was excised and homogenised (24 gels in total) in an equal volume of Freund's complete or incomplete adjuvant. Rabbits were initially injected (intracutaneously, 0.1ml/site) with extracts containing Freund's complete adjuvant (Difco Laboratories) followed by two more injections at monthly intervals with extracts containing Freund's incomplete adjuvant (Difco). Fifteen days after the last injection about 40ml of blood was taken from an ear vein, allowed to clot and then centrifuged at 2000rpm for 15 minutes. The supernatant was centrifuged again (12,000rpm) and the resulting antisera stored at -20°C until required.

4.2.6 Radial immunodiffusion

Radial immunodiffusion was by the method of Lewis and Gibson (1978). Agarose gels (1.5%) were made in 100mM sodium phosphate buffer (pH7.5) with 2.5% crude ADH antisera. Immunodiffusion was carried out for 40 hours at 4°C and the ADH/anti-ADH immunoprecipitate was stained specifically with the ADH stain described for electrophoresis. Four plates, each containing 63 wells (10ul of sample in each well) had a standard Adh^F dilution series (7 wells; 2 x 100, 50, 25 and 1 x 12.5 dilutions). The results of this standard series were found to be comparable between plates with a least square regression line fitted to circle diameter versus log concentration plots. All sample protein concentrations, expressed as arbitrary protein units, were determined from these lines and are relative to the standard series for each plate.

4.2.7 Ethanol tolerance

The ethanol tolerance of homozygotes for 11 null and two active *Adh* alleles isolated from the 1983 and 1984 samples, together with the EMS-induced allele Adh^{n10} was tested on 0, 3, and 6 percent ethanol supplemented media. Males and females were tested separately with two bottle replicates, where possible, of 20 four to eight day old adults being placed on the ethanol media at 21°C. The number dead was recorded after one and six days. Two null alleles, Adh^{nAH41} and Adh^{nAH108}, were not tested as homozygotes, but as heterozygotes with a standard Adh^F allele carried by the second chromosome balancer *Cy0*. In addition, five fertilised females of the homozygous viable null strains were placed singly in vials on 0, 3, and 6 percent ethanol media and kept at 21°C. After one and six days the number of dead females was recorded and after 24 days the number of vial cultures producing F1 progeny was scored.

4.3 Results

4.3.1 ADH activity and interallelic complementation

None of the homozygotes for the six 1983 null alleles had detectable ADH activity as adults or 3rd instar larvae. In contrast, ADH activity was detected in 1/20th and 1/30th dilutions of adult Adh^S extracts (3.5 and 3.0 activity units) and 1/50th dilutions of larvae Adh^S extracts (1 activity unit). Under normal assay conditions with an assay volume of 20ul these Adh^S adult and larval extracts were found to have mean ADH activities of 74 and 46 activity units respectively. No endogenous background reduction of NAD was detected in Adh^S adult extracts with 0.1M pyrazole.

No interallelic complementation (i.e., ADH activity) was found in the F1 heterozygotes between Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, and Adh^{nAC80} or between Adh^{nAC22}, Adh^{nAP81}, and Adh^{nAT33}. No heterodimers with residual activity were seen after electrophoresis in heterozygotes between these six alleles and Adh^{n11}, although they did occur in the Adh^{n7}/Adh^{n11} control heterozygotes (Fig. 4.1). The observed 'ultra-fast' mobility of the Adh^{n7}/Adh^{n11} heterodimer was in agreement with the known 'ADH-D-like' mobility of ADH-n11 homodimers (Schwartz and Sofer, 1976).

4.3.2 Heterodimer formation

None of the 1983 Tasmanian nulls or Adh^{n10} produced active heterodimers when heterozygous with either the Adh^F or Adh^S standard alleles (Fig. 4.2). In contrast, Adh^{n11}/Adh^F and Adh^{n11}/Adh^S heterozygotes tested on the same cellulose acetate membranes were found to produce active heterodimers.

4.3.3 ADH activity and ADH protein in heterozygotes

ADH activity and ADH protein amounts were determined in null/active allele heterozygotes for the six 1983 Tasmanian null alleles and Adh^{n10}. The activity and protein data obtained from the reciprocal crosses were combined as significant differences were only found in ADH activity for Adh^{n10}/Adh^{S} heterozygotes ($t_5 = 5.36$, $p < 0.01$) and in ADH protein for Adh^{nAC14}/Adh^{F} heterozygotes ($t_5 = 2.76$, $p < 0.05$).

Figure 4.3 shows the ADH activity and protein amounts found in the null/active allele heterozygotes. The control values for the Adh^{F}, Adh^{S} homozygotes and the Adh^{F}/Adh^{S} heterozygotes are listed in Table 4.1, from which the expected activity and protein levels of heterozygotes with one active copy of the *Adh* gene were calculated. Comparison of the heterozygote data to the expected values indicated that Adh^{nAC80}/Adh^{F} heterozygotes had significantly increased amounts of ADH activity ($t_5 = 2.93$, $p < 0.05$), as did heterozygotes between Adh^{S} and Adh^{n10}, Adh^{nAC14}, Adh^{nAC22}, and Adh^{nAC53} (Fig. 4.3). All other null/active allele heterozygotes tested had activity levels within the range expected for individuals with a single copy of the *Adh* gene. ADH protein levels in heterozygotes between Adh^{nAC14}, Adh^{nAC53}, Adh^{nAT33}, and Adh^{S} alleles were significantly higher than the expected half value of a normal Adh^{S} homozygote (Fig. 4.3). In contrast, significantly lower levels of protein were found in individuals heterozygous for Adh^{F} and the null alleles Adh^{nAP81} ($t_3 = 3.58$, $p < 0.05$) and Adh^{nAT33} ($t_3 = 3.87$, $p < 0.05$). However, none of the protein levels increased significantly without a concomitant rise in ADH activity (Fig. 4.3).

A significant difference was found in ADH activity between Adh^{nAC14}/Adh^{S} and Adh^{n10}/Adh^{S} heterozygotes ($t_{13} = 4.40$, $p < 0.001$) while protein levels in Adh^{nAC14}/Adh^{S} and Adh^{nAT33}/Adh^{S} heterozygotes

were greater than those detected in Adh^{n10}/Adh^{S} ($t_{9,7}$ = 2.80 and 4.89, p < 0.05 and 0.01 respectively).

Overall, no significant heterogeneity was detected in either ADH activity or ADH protein amongst the Tasmanian null/Adh^{F} heterozygotes (ANOVA $F_{30/5}$ = 1.19, p > 0.05; $F_{30/5}$ = 1.83, p > 0.05) or amongst the Tasmanian null/Adh^{S} heterozygotes (ANOVA $F_{5/21}$ = 1.19, p > 0.05; $F_{5/21}$ = 1.46, p > 0.05).

Figure 4.4 shows the relationship between ADH activity and ADH protein for the controls and the null/active allele heterozygotes including Adh^{n10}. Regression lines describing these relationships were fitted by the least squares method and are listed in Table 4.2. The regression of ADH protein on ADH activity for the Adh^{F} homozygote class was not significant (t_{6} = -0.19, p > 0.05). The remaining classes, including the combined Adh^{F} homozygote and Adh^{F}/Adh^{S} heterozygote class, were all found to have a significant linear component. As activity and protein appeared to be similarly related in Adh^{F} and Adh^{F}/Adh^{S} individuals (Fig. 4.4) the regression equation for the combined data was used for subsequent comparisons. Regression coefficients for Adh^{S} homozygotes and the Adh^{F} and Adh^{F}/Adh^{S} individuals were found to be significantly different (t_{13} = 4.18, p < 0.01) with Adh^{S} homozygotes having a greater increase in protein for a given increase in activity. Similarly, there was a significant difference between the two regression lines for Adh^{n}/Adh^{F} and Adh^{n}/Adh^{S} heterozygotes (t_{52} = 2.31, p < 0.05) with null/active allele heterozygotes showing activity-protein relationships characteristic of the active allele they contain. Consequently, no significant differences in regression coefficients were found between Adh^{n}/Adh^{S} and Adh^{S} homozygotes (t_{36} = 1.26, p > 0.05) nor between the Adh^{n}/Adh^{F} and the Adh^{F} and Adh^{F}/Adh^{S} lines (t_{60} = 0.86, p > 0.05).

4.3.4 Ethanol tolerance

The numbers of female and male flies dead after one and six days on ethanol media are shown in Tables 4.3 and 4.4. Male and female replicate totals were pooled and the percentage mortality of the strains for each ethanol concentration was calculated (Table 4.5). The control Adh^F and Adh^S alleles (Adh^{AC5} and Adh^{AC8} were used) show low mortality at all ethanol concentrations, although the Adh^S homozygotes are more tolerant to ethanol in these tests than the Adh^F homozygotes. The two null alleles tested as null/Adh^F heterozygotes showed mortalities similar to the Adh^F homozygote control. In contrast, homozygotes for each of the naturally-occurring null alleles had close to 100% mortality after one or six days on 6% ethanol media. Adh^{n10} showed a similar pattern of mortality on 6% media, but differed markedly from the natural nulls in having a much higher mortality on 3% media after either one or six days (Table 4.5). Adh^{n10} was unusual in having a sex-related mortality after one day on 3% ethanol media; 32 out of the 40 male flies were dead compared to only 8 of the females (Table 4.3). After six days female and male flies had similar mortalities (Table 4.4). The reason for this sex-related mortality is not known.

The difference that existed in adult mortality between Adh^{n10} and the Tasmanian nulls on the 3% ethanol media was not apparent in the vial cultures of fertilised females (Table 4.6). However, at ethanol concentrations of 6% similar levels of mortality were observed. Adh^{nAC53}, Adh^{nAC80}, and Adh^{nAT265} were exceptions to this observation in having both a low mortality and an ability to produce progeny on the 6% media. The other natural null homozygotes together with Adh^{n10} did not produce any progeny on 6% ethanol media, although all could breed successfully at the 3% concentration (Table 4.6).

4.4 Discussion

Spectrophotometric analyses of the 1983 Tasmanian null homozygotes failed to detect ADH activity either in adults or 3rd instar larvae. Under the same conditions ADH activity was detected in a 1/50th dilution of a standard Adh^S homozygote extract. Accordingly, ADH activity, if present at all, must be at levels less than 1/50th of a standard Adh^S homozygote i.e., less than about one to two activity units. Such low levels of activity would be of very limited physiological significance for an enzyme that normally comprises 1 to 2% of the total soluble protein in mature adults (Benyajati *et al.*, 1980).

The sensitivity of the enzyme assay was relevant to the $null_1/null_2$ heterozygote experiments, as interallelic complementation was to be detected on the basis of ADH activity. For a dimeric enzyme like ADH, interallelic complementation is said to occur when an active or partially active heterodimer is formed by the combination of two mutant subunits (Fincham, 1966). These subunits would normally form defective homodimers with no activity. No activity was detected in any of the 1983 $null_1/null_2$ heterozygotes and this may be due to a defective or unstable heterodimer or to the inability of the subunits to combine. Alternatively, the natural nulls may not produce any ADH protein. These explanations also apply to the lack of an active heterodimer in Adh^{n11}/null heterozygotes. Adh^{n11} is an EMS-induced null which produces a protein differing from Adh^S by a glycine-14-to-aspartic acid substitution (Thatcher, 1980). This allele is able to form active heterodimers with Adh^F or Adh^S and heterodimers of residual activity with the other EMS-induced alleles Adh^{n5}, Adh^{n6}, Adh^{n7}, and Adh^{n12}, all of which produce an inactive ADH protein (Schwartz and Sofer, 1976).

As no activity was detected in the heterozygotes these complementation experiments cannot identify different mutations. Although formally belonging to the same complementation group, the actual defect responsible for the phenotypes in the separately extracted null alleles may be quite different e.g., nonsense, frameshift, or control mutations could all have the same effect. Questions concerning the nature and hence the number of different null alleles are best resolved by molecular and protein studies (Chapters Five to Seven). However, it is worth noting that these complementation tests have provided further evidence that the loss of ADH activity is due to a defect at, or very close to, the *Adh* structural gene.

The 1983 null alleles could not form an active heterodimer with either the Adh^F or the Adh^S standard alleles, although active heterodimers were detected in Adh^{n11}/Adh^F and Adh^{n11}/Adh^S heterozygotes at positions consistent with the known ultra-fast Adh^D-like mobility of Adh^{n11} (Schwartz and Sofer, 1976). Generally, ADH activity levels and protein amounts in the null/active allele heterozygotes were within the range expected for individuals with a single copy of an active *Adh* gene. Dosage dependency is characteristic of autosomal loci in *Drosophila* (Grell, 1962; Glassman, Karam and Keller, 1962; Glassman, 1965) and has been successfully used to map loci by the use of segmental aneuploids (Rawls and Lucchesi, 1974; Hodgetts, 1975; O'Brien and Gethmann, 1973). A number of studies on several enzyme systems have observed a reduction of about 50% in enzyme activity (Hubby and Forrest, 1960; Dickinson, 1970; O'Brien and MacIntyre, 1972; Wright, Bewley, and Sherald, 1976; Schwartz and Sofer, 1976) and enzyme amount (Girton, Lo, and Bell, 1979; Bewley, deZurik, and Pagelson, 1980) in null/active allele heterozygotes and null/deficiency hemizygotes as compared to the appropriate homozygote controls.

In the present study, there were some significant departures from the expected activity and protein values calculated by assuming dosage dependency. However, at no time did ADH protein levels increase above expected levels without a corresponding increase in ADH activity, suggesting the increased protein amounts were being encoded by the active allele. Genetic background was not controlled in these experiments and could account for the observed differences. A number of studies demonstrating effects of modifiers on enzyme activity and protein amounts in natural populations support this interpretation (Ward, 1975; Lewis and Gibson, 1978; Laurie-Ahlberg, Maroni, Bewley, Lucchesi, and Weir, 1980; Laurie-Ahlberg and Bewley, 1983; Maroni and Laurie-Ahlberg, 1983). The lack of dosage compensation for the reduced levels of ADH activity in these null/active heterozygotes is consonant with the findings of Devlin, Holm, and Grigliatti (1982) who found no regulation of ADH activity to diploid levels in trisomic individuals, in contrast to their data for GPDH levels.

The relationship between ADH protein and ADH activity for Adh^S and Adh^F homozygotes were similar to those observed by Lewis and Gibson (1978). The similarity in the relationship between the Adh^F homozygotes and the Adh^F/Adh^S heterozygotes was presumably due to the dominant effect of the ADH-F allozyme. Data which support this explanation show that ethanol tolerance and relative fitness of Adh^F/Adh^S heterozygotes on ethanol supplemented media are equal to Adh^F homozygotes (Oakeshott, Gibson, Anderson, and Champ, 1980; Gibson and Oakeshott, 1982). The ADH protein-activity relationships of the Adh^n/Adh^F and Adh^n/Adh^S heterozygotes were characteristic of the active allele they contained and reflected both the lack of activity of the *Adh* null alleles and the lack of significant background interactions. It is worth noting that

the selection of either protein or activity for the ordinate and abscissa was considered arbitrary. There is evidence relating the difference in specific activity between ADH-F and ADH-S allozymes to the greater number of ADH-F molecules at equilibrium (Gibson, 1972; Lewis and Gibson, 1978; Anderson and McDonald, 1983; Clarke and Whitehead, 1984), but it is now believed that this activity difference is solely due to the known single amino acid difference of threonine in ADH-F to lysine in ADH-S (Dr M. Ashburner, *personal communication*).

The tolerance of the Tasmanian null and Adh^{n10} homozygotes to media containing 6% ethanol was poor, with all alleles having close to 100% mortality. A study by Vigue and Sofer (1976) on a series of EMS-induced *Adh* null alleles (including Adh^{n10}) found very similar mortalities for flies of comparable age on 6% ethanol. They had found that null survival on 6% ethanol was age-dependent, having a general trend of increasing sensitivity with advancing age; after about 90 hours mortality levels approached 100%. As the adult flies used in the present study were aged between 96 to 192 hours the influence of this effect was considered to be negligible, assuming it occurs in the natural null homozygotes.

The apparent difference in the response between homozygotes for natural nulls and Adh^{n10} is intriguing and merits further investigation. Admittedly, only one EMS-induced null allele was tested, but the marked difference in mortalities after both one and six days may reflect the different histories of the strains. In contrast to the Tasmanian nulls, Adh^{n10} was derived from a laboratory strain *b. Adh^{F} cn. vg.* (Gerace and Sofer, 1972) which has not been subjected to the selection pressures imposed by natural environments. ADH is clearly implicated in the detoxification of environmental ethanol (Grell *et al.*,

1968; Kamping and van Delden, 1978), but ethanol tolerance has been shown to be independent of the ADH system (McKenzie and McKechnie, 1978; Gibson *et al.*, 1979; Gibson and Wilks, 1986). No studies selecting for ethanol tolerance in *Adh* null flies at low non-toxic ethanol concentrations (i.e., 1 to 2%) have been attempted. Ethanol concentrations at the Tasmanian population sites are unlikely to be greater than 3% (Gibson *et al.*, 1981) and it is possible that the reduced mortalities of the Tasmanian null homozygotes are due to natural selection for increased ethanol tolerance occurring in these populations before the nulls arose. The observed differential responses of the Tasmanian null homozygotes to 3% ethanol media would therefore reflect genetic heterogeneity in the loci controlling or modifying ethanol tolerance.

All null strains were capable of producing progeny on 3% media, but in general failed to breed at the 6% concentration. Adh^{nAC53}, Adh^{nAC80} and Adh^{nAT265} were exceptions in having one, two, and five cultures producing progeny on 6% media. Adh^{nAT265} progeny placed on 6% media in bottles were all dead in 48 hours and were confirmed to lack ADH activity by electrophoresis. In each of these cultures the female parent had been alive after six days and the progeny observed were probably derived from a later laying after some ethanol evaporation. Ethanol concentration in supplemented food is known to decrease substantially after several days. Gibson *et al.* (1981) found that ethanol levels in media with an initial concentration of 6% falls to about 3.5% after six days. Therefore, it is likely that the longer-surviving females were laying eggs on media containing ethanol at concentrations approaching 3%. Differential ethanol evaporation between vial and bottle cultures together with crowding effects may also explain the disparity in vial and bottle mortalities.

The tolerance of null homozygotes to ethanol will be of limited relevance as null homozygotes will be very rare (less than 0.1%). Both Adh^{n}/Adh^{F} heterozygotes tested had mortalities similar to the Adh^{F} homozygotes even though the heterozygote extracts had half the ADH activity. These results support the suggestion that ethanol tolerance is not simply related to the level of ADH activity (Middleton and Kacser, 1983).

The extent to which other enzyme systems may play a role in ethanol detoxification and metabolism is unknown. Geer, Langevin, and McKechnie (1985) believe that *Adh* null flies may have a limited ability to utilise dietary ethanol. Using ^{14}C-labelled ethanol they found that larvae of an EMS-induced null, Adh^{n2}, were able to incorporate 7 to 9% as much label into lipid as wild-type larvae and suggest that aldehyde dehydrogenase, present in both active and null strains (Garcin *et al.*, 1985), may contribute to this non-ADH degradation of ethanol. In a later study Chanteux, Libion-Mannaert, Dernoncourt-Sterpin, Wattiaux-de Coninck, and Elens (1985) found aldehyde dehydrogenase activity in Adh^{n4} (also an aldehyde oxidase null) to be nearly double that found in the wild-type controls. Whether similar mechanisms exist in the Tasmanian nulls is not known, but activity assays of aldehyde dehydrogenase, aldehyde oxidase, and pyridoxal oxidase, (Geer *et al.* 1985) may prove informative.

Table 4.1. ADH activity and protein in the Adh^F, Adh^S homozygote and Adh^F/Adh^S heterozygote controls used in the null/active allele heterozygote experiments. Standard errors are given in parentheses.

Control	n	ADH activity	ADH protein
Adh^F	8	370 (20)	89 (5)
Adh^S	11	97 (7)	47 (3)
Adh^F/Adh^S	12	191 (13)	50 (3)

Table 4.2. Regression equations describing the relationship between ADH protein and ADH activity for the control and null/active allele lines.

Genotype class	n	Least squares regression line	t test$_{(Df)}$ of regression coefficient
Adh^F/Adh^F	8	$Y = 95.97 + -0.02X$	$-0.19_{(6)}$
Adh^F/Adh^S	12	$Y = 7.57 + 0.22X$	$4.94^{***}_{(10)}$
Adh^F/Adh^F and Adh^F/Adh^S	20	$Y = 14.73 + 0.19X$	$6.91^{***}_{(18)}$
Adh^S/Adh^S	11	$Y = 2.39 + 0.46X$	$8.02^{***}_{(9)}$
Adh^n/Adh^F	44	$Y = 10.25 + 0.15X$	$3.82^{***}_{(42)}$
Adh^n/Adh^S	35	$Y = 5.23 + 0.34X$	$4.71^{***}_{(33)}$

*** $p < 0.001$

Table 4.3. Number of female and male flies dead after one day on 0, 3, and 6 percent ethanol supplemented media. Replicates for each allele contained 20 adults unless marked (†).

Allele strain	Female			Male		
	0%	3%	6%	0%	3%	6%
AC5	2	0	3	0	0	2
	1	0	0	0	0	0
AC8	0	0	0 †	0	0	0
	0	0	0 †	1	0	0
AH41/CyO	0	0	-	0	0	0
	0	0	-	0	0	1
AH108/CyO	1	0	3	0	0	1
	0	0	0	0	0	1
n10	0	4	20	0	17	20
	0	4	20	0	15	20
AC14	0	2	20	0	1	20
	0	1	20	0	6	20
AC22	1	1	20	1	1	20
	1	1	20	1	0	20
AC53	1	0	20	0	1	20
	0	1	20	0	0	20
AC80	0	0	18	0	0	20
	1	2	19	2	0	20
AC95	2	2	20	0	1	20
	-	1	20	0	0	20
AP81	0	1	20	0	1	20
	0	0	20	0	0	20
AT33	0	2	20	0	2	20
	0	0	20	0	6	20
AT240	0	2	20	0	0	20
	-	0	20	1	2	20
AT265	2	1	20	0	2	19
	-	-	-	-	0 †	20
AT340	0	0	20	1	2	20
	-	3	20	0	1	20
AH36	0	1	20	1	0	20
	-	-	20	-	0 †	20

Table 4.4. Number of female and male flies dead after six days on 0, 3, and 6 percent ethanol supplemented media. Replicates for each allele contained 20 adults unless marked (†).

Allele strain	Female			Male		
	0%	3%	6%	0%	3%	6%
AC5	3	1	4	1	2	3
	3	3	6	0	0	0
AC8	0	0	0	0	0	0
	0	0	0	0	1	1
AH41/CyO	1	1	-	0	0	0
	2	2	-	0	0	1
AH108/CyO	1	1	3	0	0	1
	1	0	0	0	0	1
n10	1	13	20	0	19	20
	0	18	20	0	20	20
AC14	0	9	20	0	1	20
	0	1	20	0	13	20
AC22	1	4	20	1	9	20
	3	2	20	1	5	20
AC53	1	1	20	1	7	20
	0	2	20	0	8	20
AC80	0	1	20	0	1	20
	2	2	20	2	1	20
AC95	2	5	20	0	7	20†
	-	3	20	0	6	19†
AP81	0	1	20	0	2	20
	0	3	20	0	2	20
AT33	0	5	20	0	9	20†
	0	4	20	0	8	21†
AT240	0	2	20†	0	1	20
	-	3	19†	1	3	20
AT265	4	1	20	0	2	20
	-	-	-	-	0	20
AT340	0	3	20	0	6	20
	-	3	20	0	5	20
AH36	0	6	20	1	3	20
	-	-	20	-	2	20

Table 4.5. Percent mortality of adult flies on 0, 3, and 6 percent ethanol supplemented media after one and six days.

Allele strain	Genotype	1 Day			6 Days		
		0%	3%	6%	0%	3%	6%
AC5	*FF*	4	0	6	9	8	16
AC8	*SS*	1	0	0	0	1	1
AH41/CyO	*Fn*	0	0	3	4	4	3
AH108/CyO	*Fn*	1	0	6	3	1	6
n10	*nn*	0	50	100	1	88	100
AC14	*nn*	0	13	100	0	30	100
AC22	*nn*	5	4	100	8	25	100
AC53	*nn*	1	3	100	3	23	100
AC80	*nn*	4	3	96	5	6	100
AC95	*nn*	3	5	100	3	26	100
AP81	*nn*	0	3	100	0	10	100
AT33	*nn*	0	13	100	0	33	100
AT240	*nn*	2	5	100	2	11	100
AT265	*nn*	5	5	98	20	5	100
AT340	*nn*	2	8	100	0	21	100
AH36	*nn*	3	2	100	3	13	100

Table 4.6. Number of females dead in five vial cultures on 0, 3, and 6 percent ethanol supplemented media together with the number of these cultures which produced progeny.

Allele strain	Number dead						Cultures with progeny		
	1 Day			6 Days			24 Days		
	0%	3%	6%	0%	3%	6%	0%	3%	6%
AC5	3	0	0	3	0	3	4	5	4
AC8	0	0	0	0	0	0	5	5	5
n10	0	0	4	0	0	5	5	5	0
AC14	0	0	2	0	0	5	5	5	0
AC22	0	0	1	1	0	5	5	5	0
AC53	0	0	3	0	0	3	5	5	1
AC80	0	1	0	0	1	2	4	4	2
AC95	0	0	5	1	0	5	5	4	0
AP81	0	0	1	0	0	5	5	5	0
AT33	0	0	3	0	0	5	5	5	0
AT240	0	0	2	0	0	5	5	5	0
AT265	0	0	0	0	0	0	5	5	5
AT340	0	1	2	0	1	5	5	4	0
AH36	0	0	3	0	0	5	5	4	0

Figure 4.1. Electrophoretic phenotypes of Adh^{n11}/null heterozygotes (4 flies per sample) together with Adh^{F} and Adh^{S} homozygote controls. From left to right: Adh^{AC5}, blank, Adh^{n11}/Adh^{n} females, Adh^{n11}/Adh^{n} males, blank, Adh^{AC8}.

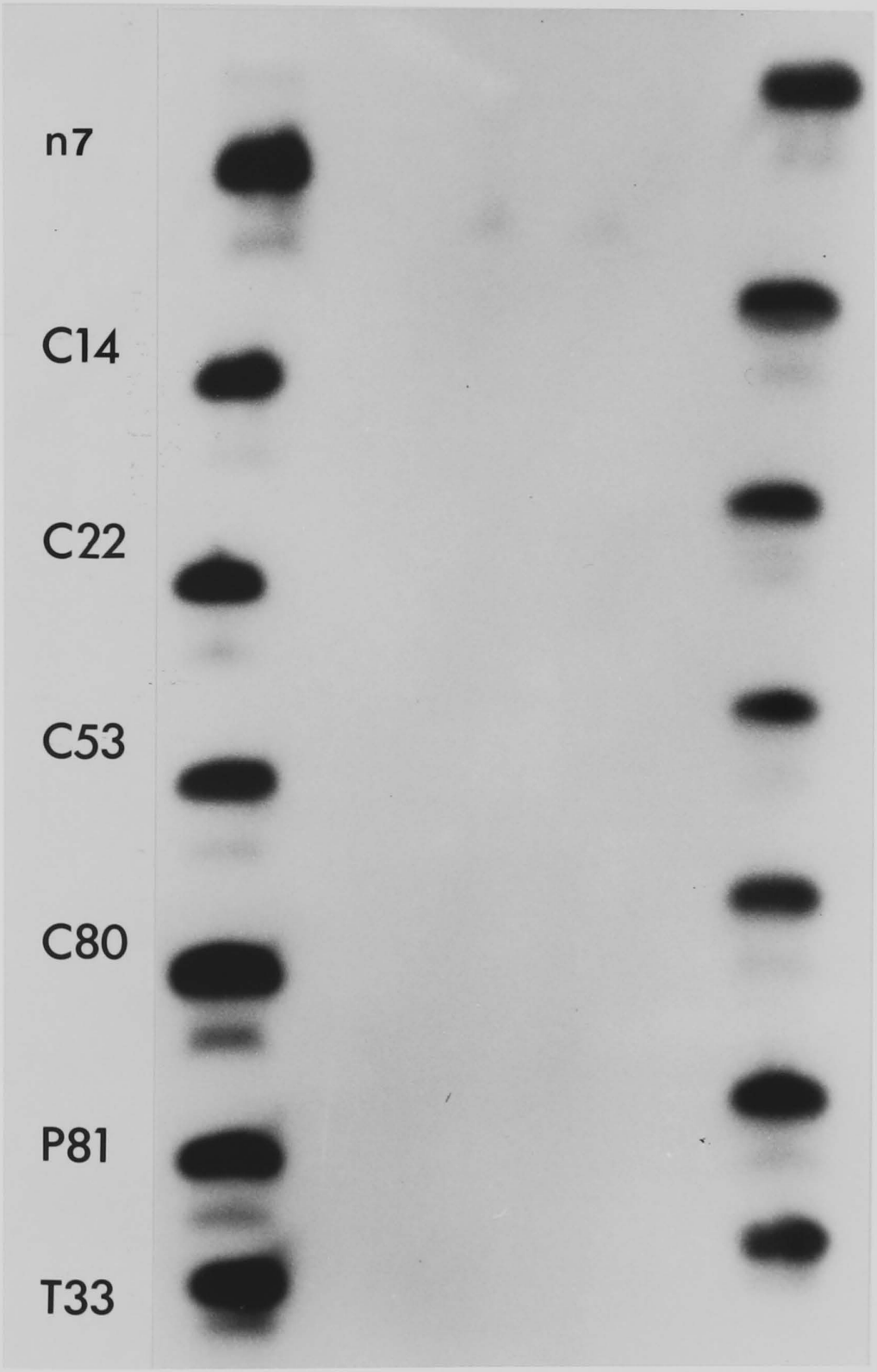

n7
C14
C22
C53
C80
P81
T33

Figure 4.2. Electrophoretic phenotypes of null/Adh^F and null/Adh^S heterozygotes together with the Adh^{n10} and Adh^{n11} heterozygote controls. From left to right: Adh^F/Adh^F, Adh^S/Adh^S, Adh^F/Adh^S, Adh^n/Adh^F female, Adh^n/Adh^F male, Adh^n/Adh^S female, Adh^n/Adh^S male.

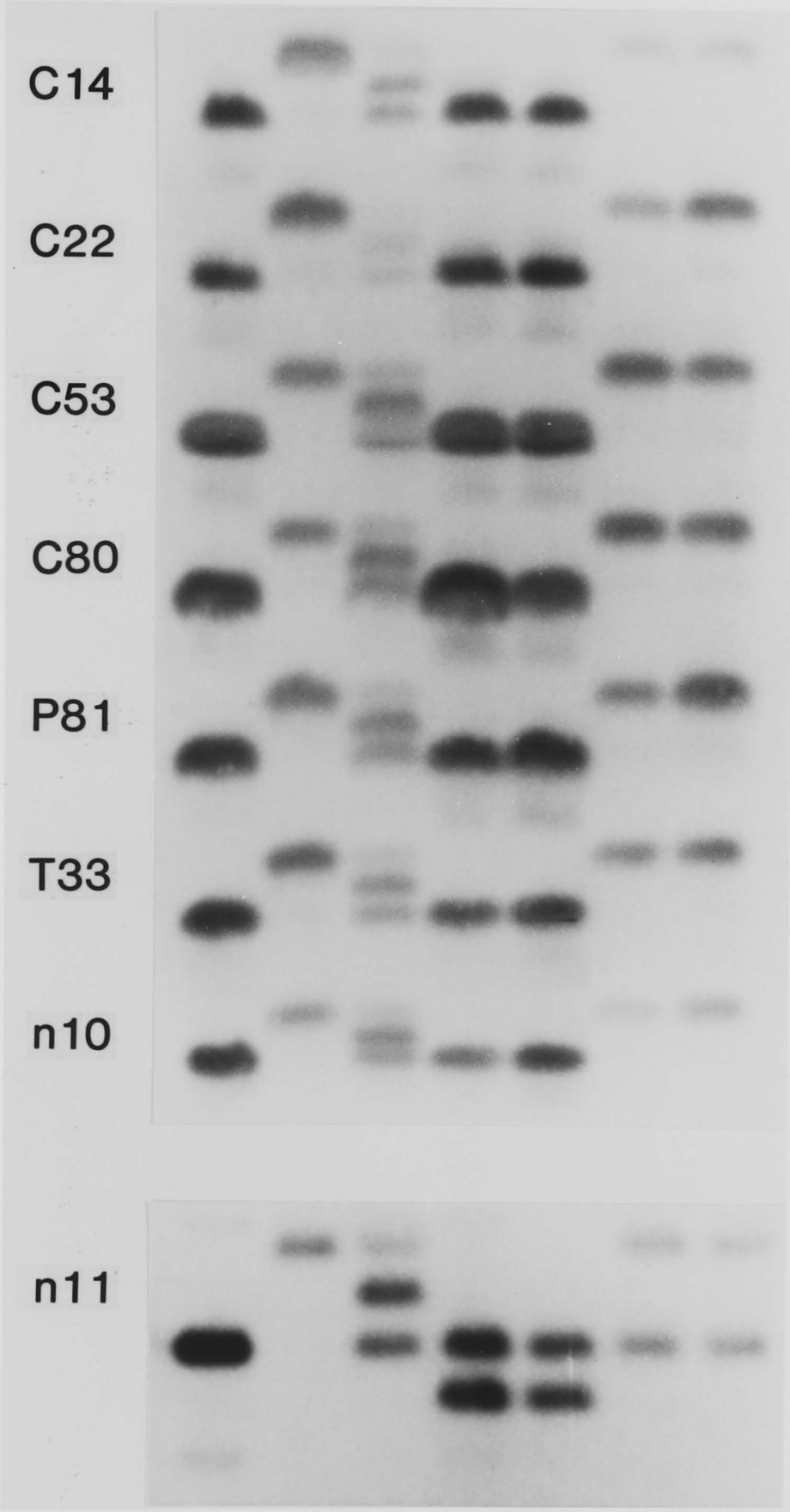
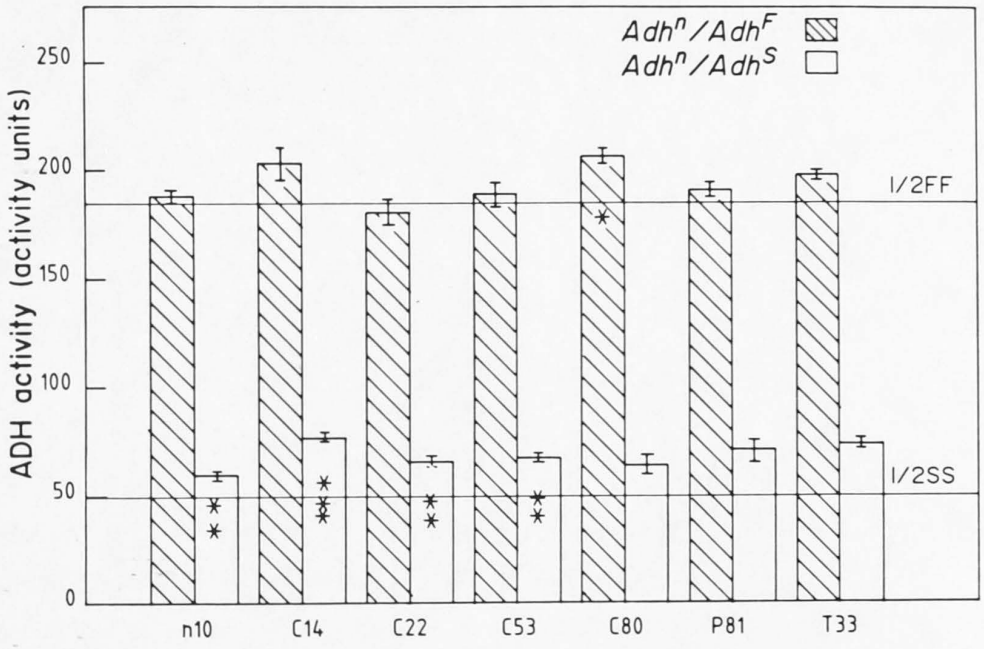

C14
C22
C53
C80
P81
T33
n10
n11

Figure 4.3. ADH activity (upper) and ADH protein amounts (lower) in Adh^n/Adh^F and Adh^n/Adh^S heterozygotes (mean ± standard error) compared to $1/2\ Adh^F/Adh^F$ and $1/2\ Adh^S/Adh^S$ levels by *t*-tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Number of extracts sampled are the same for both activity and protein measurements and are: (from left to right) *n10*, 8, 8;*AC14*, 7, 7;*AC22*, 8, 6;*AC53*, 7, 6;*AC80*, 6, 3;*AP81*, 4, 3;*AT33*, 4, 2.


Adh^n/Adh^F
Adh^n/Adh^S
ADH activity (activity units)
250
200
150
100
50
0
1/2FF
1/2SS
n10
C14
C22
C53
C80
P81
T33

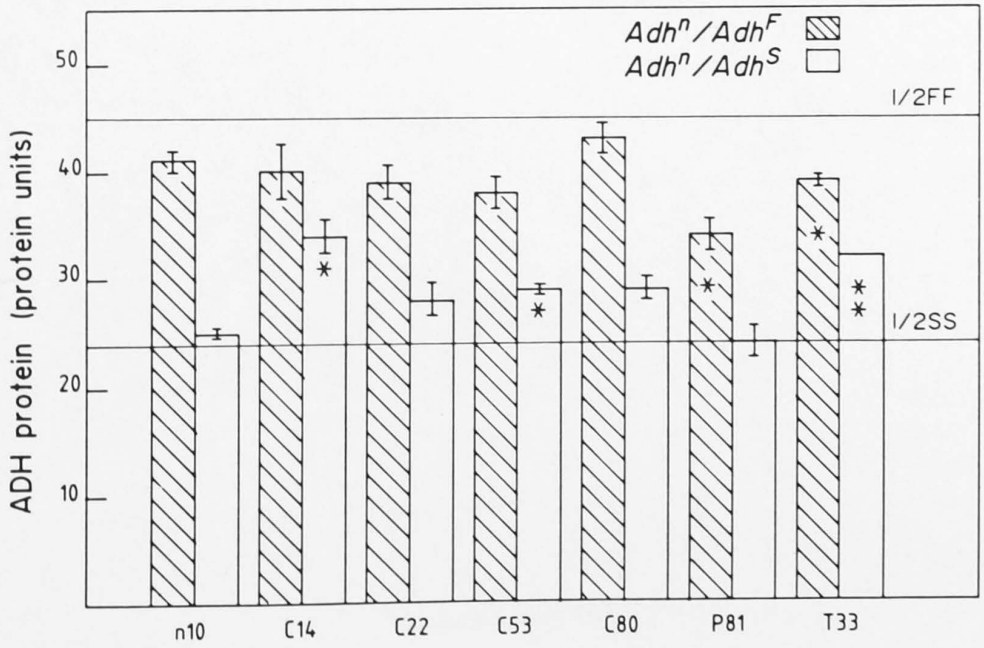
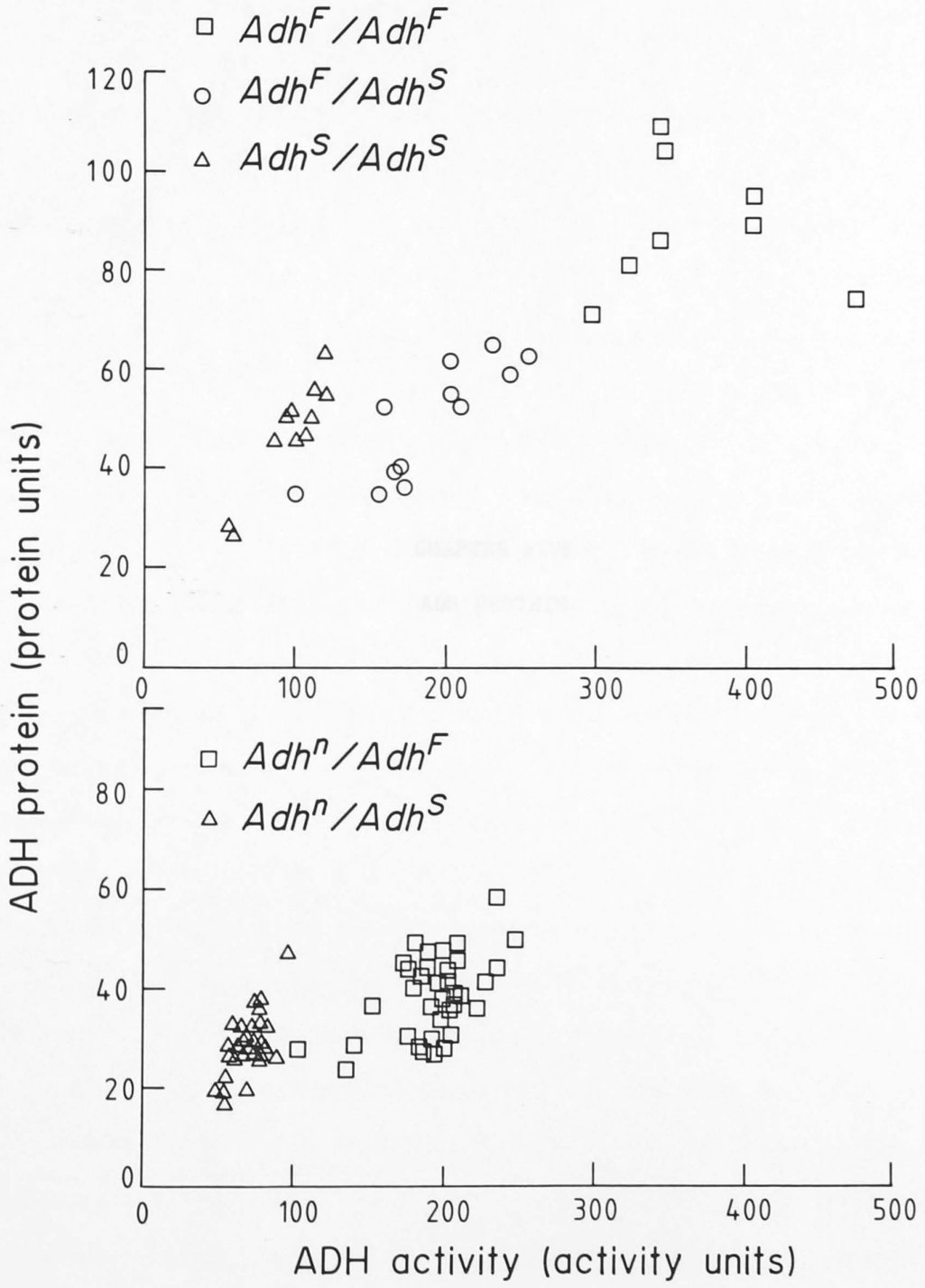

Adh^n/Adh^F
Adh^n/Adh^S
ADH protein (protein units)
50
40
30
20
10
1/2FF
1/2SS
n10
C14
C22
C53
C80
P81
T33

Figure 4.4. ADH protein versus ADH activity for the active allele controls (upper) and the null/active allele heterozygotes (lower).


□ Adh^F/Adh^F
○ Adh^F/Adh^S
△ Adh^S/Adh^S
120
100
80
60
40
20
0
0
100
200
300
400
500
□ Adh^n/Adh^F
△ Adh^n/Adh^S
80
60
40
20
0
0
100
200
300
400
500
ADH protein (protein units)
ADH activity (activity units)

CHAPTER FIVE

ADH PROTEIN

5.1 Introduction

The detection and quantification of any proteins encoded by null activity alleles is difficult due to the loss of enzymatic activity. In such cases, interallelic complementation has often been the first step to determine the presence or absence of a null gene product (Bell and MacIntyre, 1973; Wright *et al.*, 1976; Girton *et al.*, 1979); accurate quantification requires immunological and/or radiolabelling techniques.

All immunological procedures involve the preparation of an antibody against the enzyme to be investigated; generally the antisera developed are raised against partially or completely pure wild-type enzymes. The antibodies are often heterospecific, but nevertheless have significant anti-enzyme activity. Glassman and Mitchell (1959) were the first to use an immunological technique to probe for a null gene product in *Drosophila*. They determined the relative amounts of xanthine dehydrogenase (XDH) cross reacting material (CRM) by the ability of their antibody to remove XDH activity from a wild-type extract after pre-absorption with increasing amounts of null extract. If CRM was present in the null extract the XDH activity should increase as more antibody was removed by the null protein. This approach has also been used in studies of aldehyde oxidase and acid phosphatase null activity strains of *D. melanogaster* (Dickinson, 1970; Bell and MacIntyre, 1973).

The more conventional Ouchterlony and sodium dodecyl sulphate (SDS) polyacrylamide gels have been successfully used by Dickinson (1970) and Schwartz and Sofer (1976) in characterising aldehyde oxidase and alcohol dehydrogenase null mutants. However, there is always the possibility that the null allele products identified by these techniques with a non-specific stain, such as Coomassie Blue, are unrelated proteins of similar size and character. Several workers have been able to overcome

this problem by adding known amounts of an active enzyme extract as a carrier-marker. Meidinger and Williamson (1978) used the Laurell rocket technique to quantify aldehyde oxidase levels in deficient individuals by the addition of a small amount of a wild-type extract to visualise the rockets. Girton *et al.* (1979) and Browder, Wilkes, and Tucker (1982) also demonstrated the utility of this approach in studies of *D. melanogaster* null mutants and by control experiments rocket height was shown to be proportional to the total CRM present.

Early attempts to quantify the amount of CRM in the Tasmanian *Adh* nulls using similar techniques proved unsuccessful. For these experiments the antibody was raised in rabbits against partially purified ADH (see Materials and Methods). Antibody titration experiments as used by Glassman and Mitchell (1959) gave ambiguous results with inconsistent differences between control and experimental incubations. Radial immunodiffusion tests with this antiserum and using a wild-type extract as a carrier-marker were unable to differentiate two EMS-induced *Adh* nulls reported to differ by 50% in CRM amounts.

The antiserum used in these experiments had been adequate for ADH protein measurements of active allele homozygotes and null/active allele heterozygotes as described in Chapter Four. Those experiments had suggested that if the nulls produced any ADH protein, then it was present at low levels, as in general the null/active heterozygotes had levels of CRM which corresponded to half the diploid level. If the rabbit antiserum had low anti-ADH activity, then non-ADH antibody background reactions could confuse comparisons between low CRM variants.

To overcome any sensitivity problem the enzyme-linked immunosorbent assay (ELISA) technique of Engvall, Jonsson, and Perlmann (1971) was adopted. This technique detects the antigen-antibody reaction through

the use of an immunoglobulin-enzyme conjugate which reacts with multiple molecules of the appropriate substrate. The substrate is generally converted to a coloured product that can be measured spectrophotometrically. Reactants are attached to a solid phase, typically plastic tubes or cell culture plates. Use of a solid phase allows the removal of unbound reactants at each step, so that the extent of substrate conversion is proportional to the amount of antibody complexed to the plate-bound antigen (Quinnan, 1984).

The initial ELISA experiments descibed in this Chapter used the rabbit antisera and confirmed what was suspected from the titration and immunodiffusion trials; the rabbit antiserum did not contain adequate anti-ADH activity to characterise the null mutants. Professor W. Sofer provided a goat antiserum raised against purified ADH with sufficient anti-ADH activity for both the ELISA and the protein blotting experiments.

Essential for the estimation of protein levels by the ELISA technique are control strains producing known amounts of CRM. Fortunately, *Adh* is a well-studied gene-enzyme system with a large number of artificially-induced null alleles. Two EMS-induced nulls, Adh^{n7} and Adh^{n10}, were chosen for testing because Pelliccia and Sofer (1982) had estimated their CRM to be 54% and 0% of wild-type respectively.

The ELISA and Western blotting experiments characterising the Tasmanian null alleles for ADH cross-reacting material are described in this Chapter and are discussed in relation to the mutational events which could be responsible for the loss of ADH activity.

5.2 Materials and Methods

5.2.1 *Drosophila* strains

The origins of the Tasmanian *Adh* null alleles used in these experiments together with the EMS-induced nulls and active allele controls, have been described in Chapters Three and Four. For these experiments I used extracts of six null alleles isolated in 1983 (Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAC80}, Adh^{nAP81}, Adh^{nAT33}), five isolated by Dr J.B. Gibson in 1984 (Adh^{nAC95}, Adh^{nAT240}, Adh^{nAT265}, Adh^{nAT340}, Adh^{nAH36}), and one isolated by Dr J.B. Gibson in 1985 (Adh^{nAH98}).

5.2.2 Enzyme-linked immunosorbent assay

5.2.2.1 Extract preparation

Mass cultures of homozygotes for the six viable null alleles from the 1983 collections and Adh^{nAC95}, Adh^{nAT240}, Adh^{nAT265}, Adh^{nAT340}, and Adh^{nAH36} from the 1984 samples, plus Adh^{nAH98} from 1985 were set up for ADH protein assays. Contamination was checked for in the flies used to initiate the 1983 null allele cultures by pentenol vapour screening. Also set up were control cultures homozygous for Adh^{n7} and Adh^{n10}.

The preparation of extracts was modified from Gibson *et al.* (1980). Male flies aged between four and ten days were homogenised in ground glass tissue grinders in 100mM sodium phosphate buffer (pH7.5) containing leupeptin (0.02ug/ml), sodium azide (0.02%), aprotinin (0.02mg/ml), trypsin inhibitor (0.01%), phenylmethylsulfonyl fluoride (0.01mM) and Ep475 (0.1mM, Taisho Pharmaceutical Company) to a concentration of 20mg/ml fresh weight. Following homogenisation extracts were centrifuged for 30 minutes at 12,000rpm (Sorvall SM-24 rotor) to remove debris. Supernatants were filtered through Whatman GF/A glass microfibre filters and then frozen at -70°C until required.

On thawing, the samples were centrifuged for 5 minutes (15,000rpm, Eppendorf Microfuge 5414S) and the supernatants used immediately for protein assay.

5.2.2.2 ADH protein assay

Early experiments used an ADH antibody raised in rabbits by the method described in Chapter Four. This antibody had been partially purified by the absorption method of Arnheim and MacIntyre (1976). One volume of the crude ADH antibody was mixed with a half volume of an ADH-negative extract (Adh^{n10}) with phenylmethylsulfonyl fluoride (0.04mM) and bovine serum albumin (3mg/ml) and left overnight at 4°C. Next day the supernatant was passed over a CM Affi-Gel Blue column (Bio-Rad) and stored at 4°C with 1% Merthiolate (Sigma). Later, a purified ADH antibody raised in goats was used and the procedure described below refers to the experiments using this goat antibody.

The enzyme-linked immunosorbent assay (ELISA) was modified from Engvall *et al.* (1971). Purified goat ADH antibody was a gift from Professor W. Sofer. Titertek microplates (Flow Laboratories) were first activated with Polybrene (1mg/ml, Sigma) for 1 hour at room temperature, rinsed with water and 50ul of the test extracts added to the plate wells. The plates were incubated overnight at 4°C and next day, each plate was rinsed with phosphate-buffered saline containing 0.5% Tween (PBS-Tween) and blocked with 50ul/well of BLOTTO (Johnson, Gautsch, Sportsman, and Elder, 1984; 5% skim dry milk with 0.02% NaN_3 in PBS) for 1 hour at room temperature. After washing with PBS-Tween, a geometric dilution series from 1/8 to 1/16384 of the primary ADH antibody (diluted with 10% foetal calf serum [FCS], 0.02% NaN_3 in PBS) was added to each plate, 50ul per well, and incubated, shaking at room temperature for 4 hours. The washing and blocking steps were repeated before 50ul of the

secondary alkaline phosphatase-labelled goat IgG antibody (Kirkegaard and Perry Laboratories; dilution 1:100 with 10% FCS, 0.02% NaN_3 in PBS) was applied.

Following overnight incubation, plates were washed with PBS-Tween and 100ul of p-nitrophenyl phosphate (1mg/ml, Sigma) in diethanolamine buffer, pH9.8, was added to each well. After 30 minutes the reaction was stopped by addition of 50ul of 4M NaOH. For absorbance measurements 25ul of this reaction mixture was diluted with 200ul of diethanolamine buffer in flat bottom microplates and absorbance values determined at 405nm by a Titertek Multiskan MC plate reader.

5.2.3 Western blotting

Thirty male flies aged between four and ten days were homogenised in 50ul of sodium dodecyl sulphate (SDS) electrophoresis buffer (0.025M Tris, pH8.3, 0.192M glycine and 0.1% SDS) by a perspex pestle in 1.5ml microfuge tubes (Eppendorf). These extracts were boiled for 1 minute followed by centrifugation for 3 minutes at 15,000rpm (Eppendorf Microfuge) and the supernatants used immediately for SDS electrophoresis. SDS electrophoresis was carried out in the discontinuous buffer system of Laemmli (1970) using an SDS polyacrylamide gradient gel of 7 to 15% with a 4% stacking gel and the electrophoresis buffer described. Electrophoresis was at 28mA until the tracking dye (bromophenol blue) had migrated through the stacking gel and thereafter, 35mA for 4 hours. Electrophoretic transfer of proteins from the polyacrylamide gel to a nitrocellulose membrane (Schleicher and Schull BA85) was as described by Towbin, Staehelin, and Gordon (1979) using a Bio-Rad Trans Blot apparatus run overnight at 150mA with a Tris glycine buffer (pH8.3) containing 20% methanol. Next day the section of the membrane with the molecular weight markers (Bio-Rad, SDS-PAGE

Standards-low molecular weight) was stained with Coomassie blue and destained in 50% methanol with 10% acetic acid. The remainder of the nitrocellulose was blocked with BLOTTO for 1 hour, washed with PBS-Tween and incubated with the primary ADH antibody (Dilution 1:50 with 10% FCS, 0.02% NaN_3 and 3mg/ml bovine serum albumin [BSA] in PBS) in a sealed plastic bag, shaking for 4 hours at room temperature. The membrane was washed with PBS-Tween and the blocking step repeated before incubation with the secondary alkaline phosphatase-labelled anti-goat IgG antibody (dilution 1:100 in 10% FCS containing 0.02% NaN_3 in PBS) for 30 minutes at room temperature then overnight at 4°C. Next day, the filter was washed (PBS-Tween) and stained by naphthol AS-Bl phosphate (1mg/ml) and Fast Red TR salt (1mg/ml, Sigma) in 0.1M Tris-HCl (pH8.9). The staining reaction was stopped after 1 to 2 minutes by washing the membrane with water.

5.3 Results

The ADH antiserum raised in rabbits was found to be unsuitable for the ELISA technique, as the ELISA curves of absorbance versus primary ADH antibody produced by this antiserum for Adh^F and Adh^{n10} homozygotes were not separated (Fig. 5.1). This lack of separation was believed to be due to non-ADH background reactions resulting from the antiserum's lack of specificity. Similar tests with the goat ADH antiserum showed that wild-type and Adh^{n10} ELISA curves were widely separated (Fig. 5.1). Therefore, all measurements of the natural nulls were made in experiments using the goat antiserum.

ELISA curves for a single plate with the six 1983 natural nulls are shown in Figure 5.2. These alleles together with the five 1984 nulls and the single 1985 allele were tested in three experiments, each with

four plates. The two EMS-induced null alleles Adh^{n7} and Adh^{n10} were tested as controls and, as expected, differed from each other in ELISA curves (Fig. 5.2). The curves for the twelve natural nulls clustered near and around the line for Adh^{n10}.

ADH cross-reacting material (CRM) was calculated from these curves for each plate. An absorbance value in the middle of the straight line section of the curve was selected and a linear estimate of concentration for each mutant was then calculated using a least squares regression line. This concentration was converted to a percentage of wild-type by comparison with Adh^{n7}, reported to have 54% of wild-type CRM, using the Adh^{n10} data to correct for background. Mean estimates of ADH CRM for each natural mutant were obtained from the values calculated for each plate (Table 5.1). Two plates had to be excluded from the analysis because their control ELISA curves for Adh^{n10} did not cover the selected mid-point value. Similarly, one set of results for Adh^{nAC22}, Adh^{nAC80}, and Adh^{nAH98} were not used for the same reason. In most cases estimates of detectable ADH CRM were all less than 1% of wild-type levels, but for Adh^{nAC95} and Adh^{nAH36} the values were higher, although not significantly different to 1% (Table 5.1).

Nitrocellulose transfer of proteins following SDS polyacrylamide gel electrophoresis showed a major band corresponding to the 25,000 to 27,000 dalton monomer of ADH for Adh^{AC8} (an Adh^{S} allele) and Adh^{n7}. However, all the natural nulls resembled Adh^{n10} in that they lacked the presence of this major band and also several bands corresponding to polypeptides of lower molecular weight than ADH (Fig. 5.3).

5.4 Discussion

The ELISA experiments showed that the natural null alleles isolated in 1983, 1984, and 1985 had no measurable amounts of ADH cross-reacting material. The validity of this conclusion, however, is dependent on the ADH CRM-negative nature of the control Adh^{n10}. As previously described both Adh^{n7} and Adh^{n10} were selected on the basis of published reports. Pelliccia and Sofer (1982) reported values of 54% wild-type CRM for Adh^{n7} and 0% CRM for Adh^{n10}, using a radiolabelling-immunological technique which they estimated could detect CRM at levels below 1% of wild-type. More recently, Ms H. Hollocher and Dr A. Place (*personal communication*) using a variety of techniques, with both native and denatured ADH protein, have confirmed that Adh^{n10} lacks detectable CRM.

Western blotting with the ADH antibody raised in goats revealed a large number of immunoreactive protein bands common to both the control and mutant flies. These bands are probably due to both non-ADH antibody interactions caused by the heterospecific nature of the antiserum and non-antibody interactions commonly seen in Western blots of invertebrate species (Dr H.G. de Couet, *personal communication*). ADH is reported to have a subunit molecular weight of about 25,000 to 27,400 daltons (Schwartz *et al.*, 1975; Thatcher, 1980; van Delden, 1982) and a major protein band corresponding to this size range is visible in both of the controls, Adh^{AC8} (the Adh^{S} control) and Adh^{n7} which is also CRM-positive. In contrast, the natural null alleles resemble Adh^{n10} in lacking this band and also several bands corresponding to polypeptides of lower molecular weight than ADH. These smaller polypeptides are believed to arise from the degradation of the ADH monomer.

As the ELISA and Western blotting techniques are based on an antibody-antigen complex it remains possible that the Tasmanian null alleles produce polypeptides of shorter length or polypeptides which have lost their cross reactivity with the antibody. Since the antigenic determinant is believed to be in the first third of the molecule (Ms H. Hollocher and Dr A. Place, *personal communication*), defects involving early translation termination may result in the loss of antigenic reactivity. As the resolution of the Western blotting technique is believed to be around 6000 daltons (or about 60 amino acids) early termination of translation, possibly by a nonsense mutation, might result in an undetectable polypeptide. Alternatively, a point or frameshift mutation may result in the loss of the antigenic determinant without affecting polypeptide length. It is also possible that an altered but unstable polypeptide is produced.

At present, it is not known if only one mutation or if several are responsible for the lack of ADH cross-reacting material. There are a range of possible mutational events which could prevent or disrupt ADH protein production in these natural null alleles. The simplest would be a deletion removing part or all of the *Adh* gene. Smaller deletions (or insertions) could prevent initiation of transcription, RNA processing, and/or RNA translation. There are reported examples of these types of defects for *Adh* in *D. melanogaster* (Ashburner *et al.*, 1982; Benyajati *et al.*, 1983; Kelley, Mims, Farnet, Dicharry, and Lee, 1985). Such insertions or deletions may only involve one or two bases which could result in a frameshift mutation either generating a termination codon or disrupting recognition of the mRNA splice site junctions. A variation of this type of mutation has been reported by Chia, Savakis, Karp, Pelham, and Ashburner (1985) for Adh^{nLA248} in which a duplication of

parts of coding and noncoding regions of the *Adh* gene has generated a frameshift which results in termination of translation.

Alternatively, a single base substitution can result in the formation of a termination or nonsense codon. MacIntyre and O'Brien (1976) have outlined four criteria by which null alleles might be identified as nonsense mutations. They are (1) a lack of interallelic complementation, (2) an inability to form an active multimer with an active allele, (3) no detectable CRM, and (4) the presence of a polypeptide of altered size on SDS polyacrylamide gels. The Tasmanian nulls investigated do not show interallelic complementation, they appear to lack detectable ADH CRM, and they are unable to form active heterodimers with either Adh^F or Adh^S standard alleles.

Two nonsense mutations have been reported in *D. melanogaster*. Martin, Place, Pentz, and Sofer (1985) have shown that Adh^{nB}, an EMS-induced null allele which has ADH polypeptide levels reduced to 1%, a lowered rate of synthesis and increased rate of degradation, contains a base substitution changing the TGG tryptophan codon at amino acid 235 to a TGA termination codon. Similarly, Karlik, Coutu, and Fyrberg (1984) have reported another opal TGA nonsense mutation in the *Drosophila* 88F actin gene resulting in a stable truncated actin polypeptide, still antigenic to actin antibodies, but disrupting myofibril formation in the indirect flight muscles. In addition, there is some evidence that another EMS-induced *Adh* null allele, Adh^{n4} is also caused by a nonsense mutation. This null has no detectable ADH protein and has mRNA levels 5-10% that of wild-type (Drs W. Chia and M. Ashburner, *personal communication*). Whatever the mutation event(s) responsible for the Tasmanian null alleles turns out to be, protein analyses have not provided any evidence for heterogeneity between separately extracted null alleles.

Table 5.1. The amount of ADH cross-reacting material (CRM) expressed as a percentage of the amount in Adh^F/Adh^F homozygotes for twelve null alleles isolated from the Tasmanian populations in 1983, 1984, and 1985. Standard errors are given in parentheses.

Year sampled	Population	Null allele	ADH CRM 1	ADH CRM 2
1983	Cygnet	*AC14*	0.2 (0.09)	0.1 (0.06)
1983	Cygnet	*AC22*	0.5 (0.25)	0
1983	Cygnet	*AC53*	0.5 (0.15)	0.5 (0.26)
1983	Cygnet	*AC80*	0.2 (0.12)	0
1983	Pipers Brook	*AP81*	0.3 (0.11)	0.5 (0.21)
1983	Tamar	*AT33*	0.6 (0.09)	0.3 (0.15)
1984	Cygnet	*AC95*	1.8 (0.92)	-
1984	Tamar	*AT240*	0.9 (0.93)	-
1984	Tamar	*AT265*	0.7 (0.74)	-
1984	Tamar	*AT340*	0.6 (0.58)	-
1984	Huonville II	*AH36*	2.9 (1.47)	-
1985	Huonville II	*AH98*	0.9 (0.93)	-

Figure 5.1. ELISA curves of absorbance versus primary ADH antibody log concentration x 10^5 for Adh^F and Adh^{n10} homozygous strains from two separate experiments using either the rabbit ADH antiserum or the goat ADH antiserum provided by Professor Sofer. Absorbance values were determined by the difference between two measurements at 405nm and 620nm.

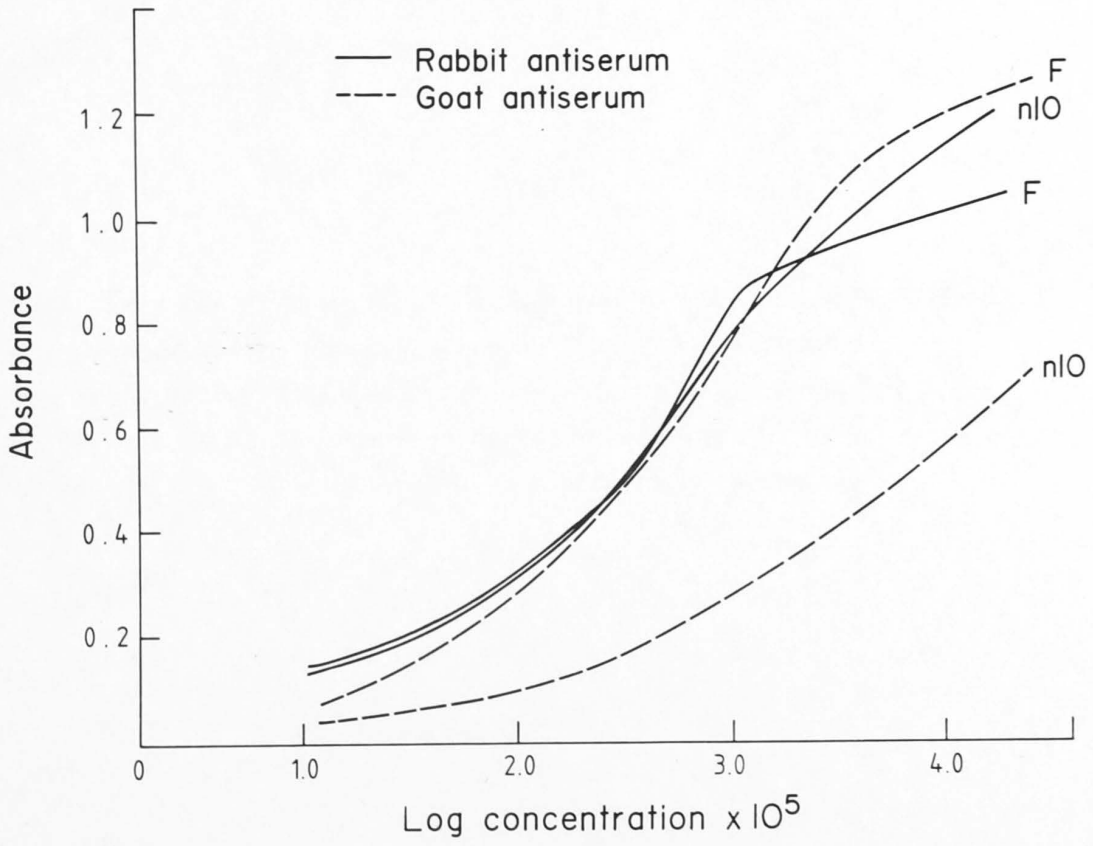

Rabbit antiserum
Goat antiserum
F
nIO
F
nIO
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
1.0
2.0
3.0
4.0
Log concentration x 10^5

Figure 5.2. ELISA curves of absorbance versus primary ADH antibody log concentration x 10^5 for one plate containing the six natural null alleles and two EMS-induced nulls, Adh^{n7} and Adh^{n10}. Absorbance values were determined by a single measurement at 405nm.

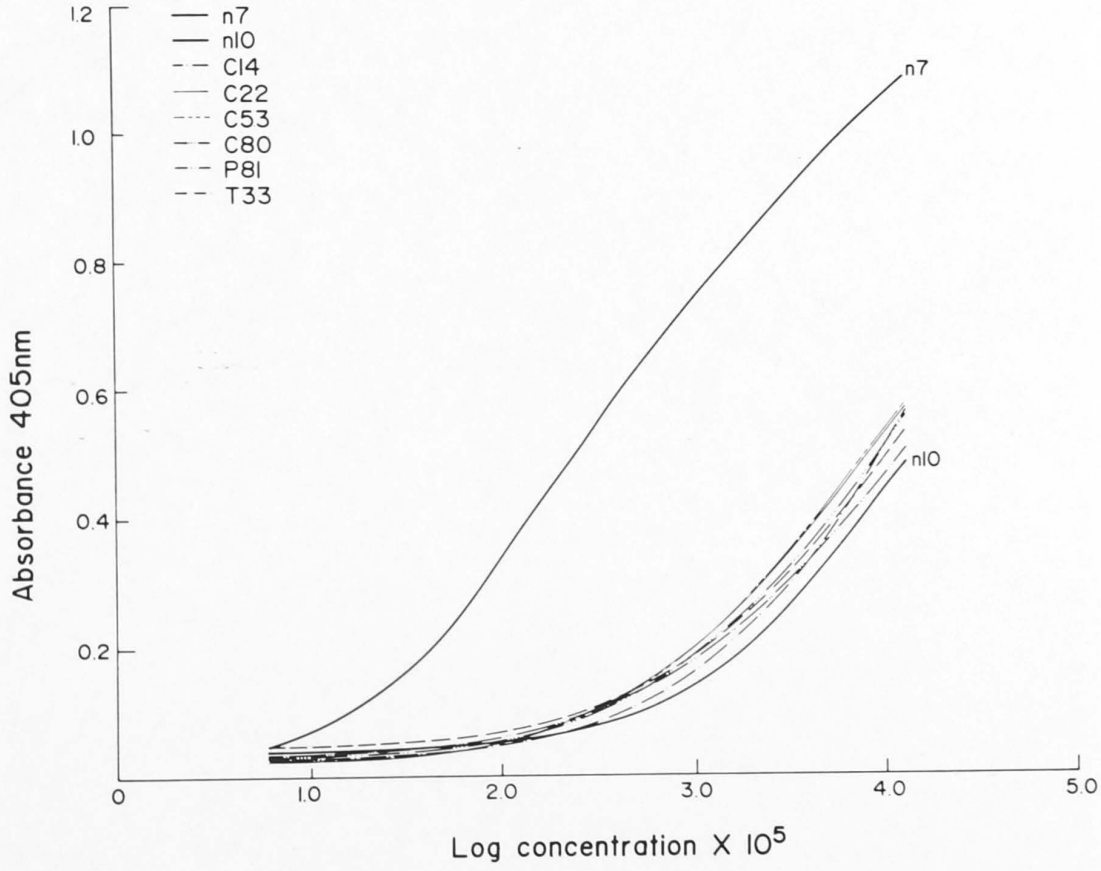
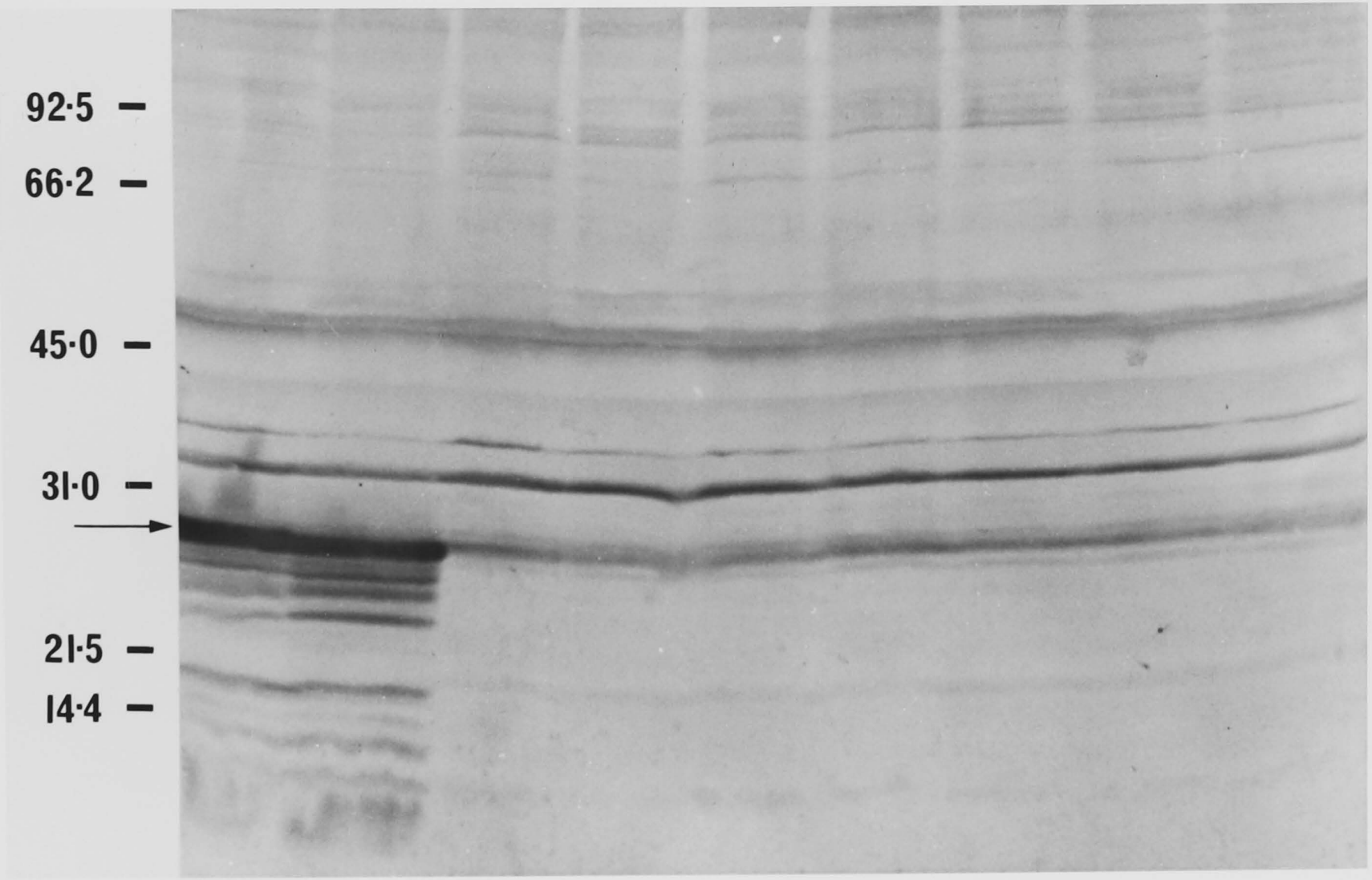

n7
nl0
Cl4
C22
C53
C80
P8l
T33
1.2
1.0
0.8
0.6
0.4
0.2
0
1.0
2.0
3.0
4.0
5.0
n7
nl0
Absorbance 405nm
Log concentration X 10^5

Figure 5.3. Nitrocellulose transfers of wild-type and mutant proteins after SDS polyacrylamide gel electrophoresis. Arrowed is the 25-27 K dalton band of ADH seen in lanes 2 and 3. From left to right: Adh^{AC8}, Adh^{n7}, Adh^{n10}, Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAC80}, Adh^{nAP81}, Adh^{nAT33}.


92·5
66·2
45·0
31·0
21·5
14·4

CHAPTER SIX

THE MOLECULAR LANDSCAPE IN THE *Adh* REGION OF THE NULL ALLELES

6.1 Introduction

The *Adh* gene of *D. melanogaster* has been cloned and sequenced (Goldberg, 1980; Benyajati, Place, Powers, and Sofer, 1981; Kreitman, 1983) and consists of three protein encoding sequences of 96 base-pairs (bp), 405 bp, and 264 bp separated by two small introns (65 bp and 70 bp). A polyadenylation signal is found 121 bp 3' to the termination codon while two TATA boxes, located 95 bp and 803 bp 5' to the initiation codon, are believed to selectively promote the transcription of the 1100-base and 1150-base mRNAs observed in second instar larvae and adults respectively (Benyajati, Spoerel, Haymerle, and Ashburner, 1983).

It is possible that the loss of ADH function in the Tasmanian nulls arises from a mutation(s) within the *Adh* gene or perhaps in adjacent *cis*-acting regulatory sequences. Although the mutation(s) responsible may affect transcription, mRNA processing, and/or translation, the first possibility to be considered is a structural alteration in the *Adh* gene itself or the surrounding molecular landscape.

All the Tasmanian *Adh* null alleles examined were homozygous viable and viable with deficiencies of the *Adh* region. However, this does not necessarily mean that deletions are not present in the *Adh* null alleles, as Ashburner *et al.* (1982) have reported deletions spanning the *no-ocelli-Adh-outspread* region to be viable both as homozygotes and as heterozygotes with the deficiency *Df(2L)64j*.

Alternatively, insertions in either the coding or noncoding regions of the *Adh* gene may disrupt ADH production. A known mechanism for the production of structural mutations is the movement of transposable elements, with either insertion or imprecise excision disrupting gene function (Green, 1980; Berg, Egner, Hirschel, Howard, Johnsrud,

Jorgensen, and Tlsty, 1981; Rubin, Kidwell, and Bingham, 1982; Zachar and Bingham, 1982). High spontaneous mutation frequencies apparently associated with mobile elements have been found in a number of natural *Drosophila* populations (Woodruff *et al.*, 1983).

In the past, investigation of structural defects at null enzyme loci has been by cytological analyses (Grell *et al.*, 1968; O'Donnell *et al.*, 1977); however, the development of recombinant DNA techniques, particularly restriction endonuclease and Southern blot analysis, has allowed smaller and more complex rearrangements and alterations to be detected (Benyajati, Place, Wang, Pentz, and Sofer, 1982; Chia *et al.*, 1985; Kelley *et al.*, 1985). This chapter describes a Southern blot analysis of an 11.8 kb chromosomal region containing the *Adh* gene from each of the homozygous viable null alleles isolated in 1983. The aim of the study was to ascertain whether the DNA in the region of the *Adh* locus in the Tasmanian null alleles was intact and free from major insertions or deletions.

6.2 Materials and Methods

6.2.1 *Drosophila* strains and culture

The six homozygous viable *Adh* null alleles and the Adh^S allele (Adh^{AC8}) isolated from the Tasmanian 1983 population collections and used for these analyses have been previously described (Chapters Three and Four).

To collect embryos for DNA analysis Adh^{nAC80} and Adh^{AC8} stocks were each expanded to 60 bottle cultures, from which adults were combined and transferred to separate egg collection cages which were approximately 1 cubic metre in size. The Adh^{nAC80} stock was tested for contamination by the pentenol vapour test prior to culture expansion. High protein food

as described in Chapter Two (without HiPro powder) was mixed with charcoal and poured into flat bottom polystyrene trays. Blackcurrant juice and yeast paste were smeared on the food surface just before the trays were placed in the collection cages. At 20 hour intervals, trays were removed and the eggs laid were collected in a muslin filter by rinsing the food surface with T.E. buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). After dechorionation with 1% bleach, embryos were stored in T.E. buffer at -70°C until required. On completion of embryo collections, the adult flies were starved for 2 hours, anaesthetised by CO_2, frozen in liquid nitrogen, and stored at -70°C.

For adult DNA analysis the Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAP81} and Adh^{nAT33} stocks were screened by pentenol vapour and then expanded on unmodified HiPro culture food. Adult flies were collected at regular intervals, starved for 2 hours, then frozen in liquid nitrogen, and stored at -70°C.

6.2.2 DNA clones

The recombinant plasmids sAC1 and sAF2 were obtained from Dr D.A. Goldberg and are described in Goldberg (1981). Plasmid sAC1 contains a 4.75 kb *Eco*RI fragment which was subcloned into pBR322 from a bacteriophage lambda (λ) library (Maniatis, Hardison, Lacy, Lauer, O'Connell, Quon, Sim, and Efstratiadis, 1978) derived from *D. melanogaster* Canton S DNA. DNA from the *Adh* region of a homozygous Adh^F strain was cloned as an 11.8 kb *Sac*I fragment into the bacteriophage vector Charon 10 and later subcloned into a pBR322-derived *Sac*I vector (pSV2) to give the sAF2 clone. pSV2 had been constructed by placing a central *Eco*RI fragment from Charon 9 into pBR322 and subsequently removing the internal *Sac*I fragments (Goldberg, 1980).

The restriction enzyme map of the *Adh* gene region as determined by Goldberg (1980) is shown in Figure 6.1, with the regions covered by the sAC1 and sAF2 clones indicated.

6.2.3 Extraction of DNA

Genomic DNA was extracted from embryos and adults by the method of Miklos (1984). Adults or embryos were frozen in liquid nitrogen and homogenised in buffer (10mM Tris, pH8.0, 20mM EDTA), lysed with the detergent Sarkosyl NL-30 (final concentration 3%), and the lysate added directly to solid caesium chloride (final concentration 1g/ml). To this mixture ethidium bromide was added to a final concentration of 0.6mg/ml and ultracentrifugation was at 40,000rpm (Ti 50 rotor) for 40 to 45 hours. The main genomic DNA band was visualised by ultra-violet (U.V.) light (366nm), collected by side puncture with a 19 gauge needle and the ethidium bromide was removed by six extractions with isopropanol. The DNA was dialysed overnight against T.E. buffer (10mM Tris-HCl, pH8.0, 1mM EDTA) at 4°C. The DNA solution was then transferred to microfuge tubes (Eppendorf) and stored at -20°C until required. The isolated DNA was checked by ethidium bromide electrophoresis of undigested DNA to make sure that it was not degraded and its concentration was usually about 60 to 100ug/ml.

6.2.4 Isolation of supercoiled plasmid DNA

LM media (bactotryptone [10g], yeast extract [5g], NaCl [10g], maltose [2g], $MgCl_2.2H_2O$ [0.2g], 10mM Tris-HCl, pH7.0, made up to 1 litre with water) was inoculated in the presence of ampicillin (50ug/ml) with *Escherichia coli* carrying the recombinant plasmids and incubated overnight at 37°C. Plasmid-containing bacteria were recovered by centrifugation in polypropylene bottles at 5000rpm (GSA rotor) for 10 minutes at 4°C. The bacterial cells were resuspended with 20% sucrose

in T.E. buffer (1.6ml) and kept on ice. Lysozyme was added (2.0mg/ml) and the suspension left for 5 minutes before the addition of 0.7ml of 0.25 M EDTA. After 2 minutes incubation, the cells were lysed with 2.4ml of lytic mix (10% Triton X-100, 1.0M Tris, pH8.0, 0.25M EDTA) for 10 minutes at 4°C and then centrifuged at 16,000rpm (SS34 rotor) for 40 minutes. The supernatant was added to solid caesium chloride (1g/ml) and ethidium bromide (0.6mg/ml). Ultracentrifugation, DNA recovery and ethidium bromide extraction was as described in Section 6.2.3.

6.2.5 DNA sample preparation

Digestion of DNA samples by restriction endonucleases was carried out under the conditions recommended by the manufacturers, except for *Hin*dIII where digestions were completed in T.A. buffer (33mM Tris-HCl, pH7.9, 66mM potassium acetate, 10mM magnesium acetate, 5mM dithiothreitol). *Eco*RI was a gift from A. McKenzie while *Hae*III and *Hin*dIII were obtained from Amersham. For genomic DNA 10 to 18ul of the DNA solution containing about 0.5 to 1.5ug of DNA (for plasmid DNA, 5 to 8ul) was added to the digestion mixture (total volume 23.3ul) and incubated for 2 hours at 37°C. The reaction was stopped by addition of 5ul of sample dye (30% sucrose, 0.09% bromophenol blue, 50mM EDTA) and stored at 4°C until required for electrophoresis.

6.2.6 Electrophoresis and Southern blotting

Electrophoresis of DNA samples was performed in a horizontal gel apparatus with 1.0% agarose (SeaKem ME) gels prepared in electrophoresis buffer (0.04M Tris-HCl, pH7.8, 5mM sodium acetate, 1mM EDTA). Electrophoresis was for 14 to 16 hours at about 35mA (30 to 40V). The gel and electrophoresis buffer contained ethidium bromide (2.5ug/ml and 2ug/ml respectively) and the DNA bands were visualised under U.V. light (254nm) and photographed using Polaroid Type 55 Land Positive/Negative

film. Lambda DNA (c1857) digested with *Hin*dIII was used to provide size markers where required. Transfer of DNA from agarose gels to nitrocellulose membranes was by the method of Southern (1975) with minor modifications. Gels were trimmed and transferred to a denaturing solution (0.8M NaCl, 0.4M NaOH) for two washes of 45 minutes followed by neutralisation by two washes of 60 minutes in 1.5M NaCl, 0.5M Tris-HCl, pH7.4. Transfer of DNA was by blotting in 20 x SSC (2.7M NaCl, 0.27M sodium citrate) for 20 to 24 hours. The nitrocellulose membrane or filter (Schleicher and Schull BA85) was washed in 2 x SSC for 5 minutes and then baked for 3 to 4 hours at 80°C under vacuum.

6.2.7 Hybridisation of Southern blots

Hybridisation probes were prepared by the random primer method using denatured calf thymus DNA. Plasmid DNA (about 0.5ug to 1.5ug) was first digested with *Hae*III enzyme and then boiled for 4 minutes in the presence of the calf thymus random primers (0.1ug) and quick-cooled on ice. A mixture of three deoxyribonucleotides (dATP, dGTP, and dTTP at about 2mM) was added together with 1.5ul of DNA polymerase 1 (large fragment, Bresa), 3ul of [α-^{32}P]-dCTP (Amersham; 10mCi/ml giving 30uCi in 3ul), and 2.2ul of *Hae*III buffer (6mM NaCl, 6mM Tris-HCl, pH7.4, 6.6mM $MgCl_2$ and β-mercaptoethanol, 4.7ul/ml) in a total reaction volume of 35ul. This reaction mixture was incubated at 37°C for 30 minutes and then, to remove the unincorporated nucleotides, put through a 3ml Sephadex-G50 (Pharmacia) column equilibrated in T.E. buffer. Radiolabelled DNA was eluted from the disposable columns by centrifugation (2000rpm for 5 minutes) and a Geiger counter reading on a 100ul sample was used to check incorporation. Hybridisation probes were stored at -20°C until required.

Prehybridisation of nitrocellulose filters was carried out in sealed plastic bags for 1 hour at 65°C in 3 x SSC containing 10 x Denhardt's solution (Denhardt [1966] : 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) with 90ug/ml of heat-denatured herring sperm DNA. Hybridisation was carried out in a 1:10 dilution of the prehybridisation solution with 3 x SSC. The radioactive DNA probe was denatured at 100°C for 4 minutes and added directly to the plastic bag containing the filter and the hybridisation solution. Hybridisation was at 65°C for 14 to 16 hours after which filters were washed in 2 x SSC at 65°C (2 x 60 minute washes) and then dried for 45 minutes at 80°C under vacuum. Autoradiography of filters was at -70°C using Kodak X-ray film (XRP-1) and DuPont Lightning Plus intensifying screens.

6.3 Results

Southern transfer of *Eco*RI-digested genomic DNA of Adh^{AC8} (control Adh^{S} line) and Adh^{nAC80} probed with the sAC1 clone revealed no differences between embryos and adults or between the active and null alleles (Fig. 6.2a). As expected (see Fig. 6.1) a single band corresponding to a molecular weight of about 4.7 kb was detected in Adh^{AC8}. This band was also present in the null strain. The lower hybridisation intensity of the adult bands was due to the lower amounts of total DNA loaded (Fig. 6.2b). As no differences were detected between adult and embryo DNA preparations all subsequent experiments used genomic DNA from adults.

*Eco*RI restriction patterns for genomic DNA of Adh^{AC8} and the six 1983 nulls after hybridisation to the sAF2 clone are shown in Figure 6.3a. The *Adh* gene region covered by the 11.8 kb sAF2 genomic probe contains five *Eco*RI sites which give five fragments of 4.75, 2.4, 1.8,

1.45, and 0.3 kb (Fig. 6.1) and another fragment of 2.4 kb comprising about the last 1.3 kb of the 3' region covered by the sAF2 clone (arrowed in Figure 6.1) and about 1.1 kb of adjoining DNA outside the region covered by this clone. This fragment was not detected as a separate band because it co-migrates with the other 2.4 kb fragment (Mr Chengshan Jiang, *personal communication*). The 0.3 kb fragment was not detected in Adh^{AC8} or in the six 1983 nulls, probably because it migrated beyond the region blotted. Similarly, the 0.56 kb *Hin*dIII fragment of the lambda (λ) DNA used for size markers was not detected.

The Southern restriction patterns of the six 1983 null alleles were identical to the active control allele Adh^{AC8} (Fig. 6.3a). As before, differences in band hybridisation intensities reflected differential loading of total DNA (Fig. 6.3b). Detailed restriction maps prepared by Mr Chengshan Jiang (*personal communication*) have shown that Adh^{AC8} has a restriction map like that of the Adh^{F} allele from which the sAF2 clone was derived (i.e., Fig. 6.1). Size estimates were obtained from migration distance of fragments as compared to the distance travelled by the lambda DNA markers. The average size of the four detectable fragments were calculated from all seven strains for this Southern transfer and were found to be comparable to those expected, with little variation between the nulls and Adh^{AC8} (Table 6.1). The value of 1.1 kb for the smallest detectable band was lower than expected, but as no 0.56 kb lambda *Hin*dIII marker was detected this value was obtained by extrapolation of the standard line relating marker size to migration distance and the estimate could be inaccurate.

6.4 Discussion

No differences were detected between the embryo and adult Southern patterns of Adh^{AC8} and Adh^{nAC80}. In *D. melanogaster* the conventional source of DNA from diploid tissues has been early embryos but more recently, studies have tended to isolate DNA from whole adults (Bingham, Levis, and Rubin, 1981; Chia *et al.*, 1985) and thereby avoided the time-consuming process of embryo collection.

Southern blot analysis of the 11.8 kb region containing the *Adh* gene did not reveal any major insertions or deletions, as compared to Adh^{AC8}, in the 1983 Tasmanian null alleles. The resolution of this technique with one restriction enzyme is limited to changes of about 200 bp and increases to about 50 bp when several enzymes are used (Zachar and Bingham, 1982). Ms A.V. Wilks (*personal communication.*) has used eight restriction six base-pair cutting endonucleases and has found no difference in the Southern restriction patterns in the *Adh* region of seven null alleles isolated from Tasmania in 1984 and seven isolated in 1985. Therefore, it is unlikely that structural alterations of greater than 50 bp are responsible for the loss of ADH activity. Certainly, there is as yet no evidence of mobile element insertion anywhere in the *Adh* region and this is consistent with the reported failure to generate P-element mutations of *Adh* by hybrid dysgenesis (Woodruff *et al.*, 1983).

Goldberg, Posakony, and Maniatis (1983) used a P-element-mediated transformation to introduce the 11.8 kb fragment of Goldberg's sAF2 clone into the germ line of *Adh* null flies. They found that this 11.8 kb chromosomal region included the *cis*-acting sequences necessary for the correct developmental expression of the *Adh* gene. Indirect evidence implicating the disruption of these sequences in *Adh* null flies was obtained by Kelley *et al.* (1985) for the spontaneous null allele,

Adh^{nLA319}. This null has altered Southern restriction patterns indicative of structural alterations in the 11.8 kb sAF2 region, but has normal restriction patterns when probed with the smaller 4.75 kb sACl clone. It remains possible therefore that a small insertion or deletion has occurred in a *cis*-acting regulatory sequence of the Tasmanian null alleles and disrupted *Adh* expression.

However, there are a series of mutations which could occur within a gene sequence that can disrupt gene function without affecting the structural integrity of a locus. Nonsense mutations, well-known in other organisms (Glass, Nene, and Hunter, 1932; Waterston and Brenner, 1978; Gorski, Fiori, and Mach, 1982) have recently been reported in *D. melanogaster* (Kubi, Schmidt, Martin, and Sofer, 1982; Karlik *et al.*, 1984; Martin *et al.*, 1985). Kelley *et al.* (1985) in an analysis of the known *Adh* sequence have identified 20 potential C to T and 14 G to A transition sites that would result in early termination. Alternatively, a frameshift mutation could result in the generation of a nonsense codon and according to Kelley *et al.* (1985) there are 12 possible nonsense codons which could result from a frameshift caused by the insertion or deletion of 1 to 2 bases, two of which are in the first ten amino acids.

Similar single or multi-base substitutions, insertions, or deletions could occur in the splice site junctions of the *Adh* transcripts affecting intron splicing and preventing subsequent translation. Such mutations have been reported in *D. melanogaster* for two formaldehyde-induced *Adh* null alleles (Benyajati *et al.*, 1982).

To distinguish the type of mutation(s) responsible for the loss of *Adh* function in the Tasmanian nulls it is necessary to know: first, if *Adh* mRNA is produced, and second, if the structure and amount of the transcript have been altered.

Table 6.1. Mean size (standard error) of *Eco*RI fragments as detected by the sAF2 clone (Fig. 6.3a) for Adh^{AC8} and the six 1983 null alleles.

Expected fragment size (kb)	n	Estimated fragment size (kb)
4.8	7	4.7 (0.02)
2.4	7	2.3 (0.01)
1.8	7	1.9 (0.01)
1.5	7	1.1 (0.01)

Figure 6.1. Restriction map of the *Adh* region as taken from Goldberg (1980). Fragment sizes after digestion with *Eco*RI and the regions covered by the sACl (4.75kb) and sAF2 (11.8kb) clones are indicated. The 5' to 3' orientation of the *Adh* mRNA is from left to right. Arrowed is about 1.3 kb of DNA covered by the sAF2 clone which is part of a 2.4 kb *Eco*RI genomic DNA fragment (see text).

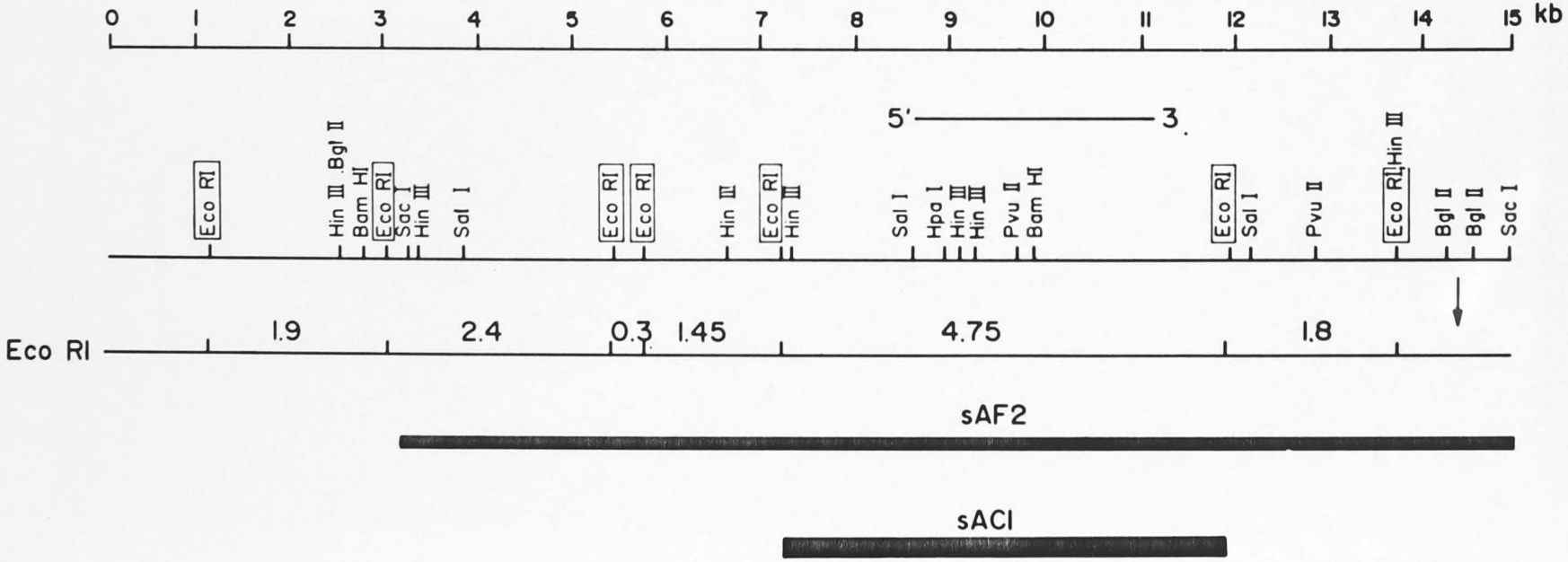

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 kb
5' 3'
Eco RI
Hin III Bgl II
Bam HI
Eco RI
Sac I
Hin III
Sal I
Eco RI
Eco RI
Hin III
Eco RI
Hin III
Sal I
Hpa I
Hin III
Hin III
Pvu II
Bam HI
Eco RI
Sal I
Pvu II
Eco RI Hin III
Bgl II
Bgl II
Sac I
Eco RI
1.9
2.4
0.3
1.45
4.75
1.8
sAF2
sACI

Figure 6.2. Southern transfer of *Eco*RI-digested genomic DNA probed with sACl DNA (a) and the digested DNA prior to transfer as visualised by ethidium bromide staining (b). From left to right: Adh^{nAC80} embryos, λ*Hin*dIII, Adh^{AC8} embryos, Adh^{nAC80} adults, Adh^{AC8} adults.

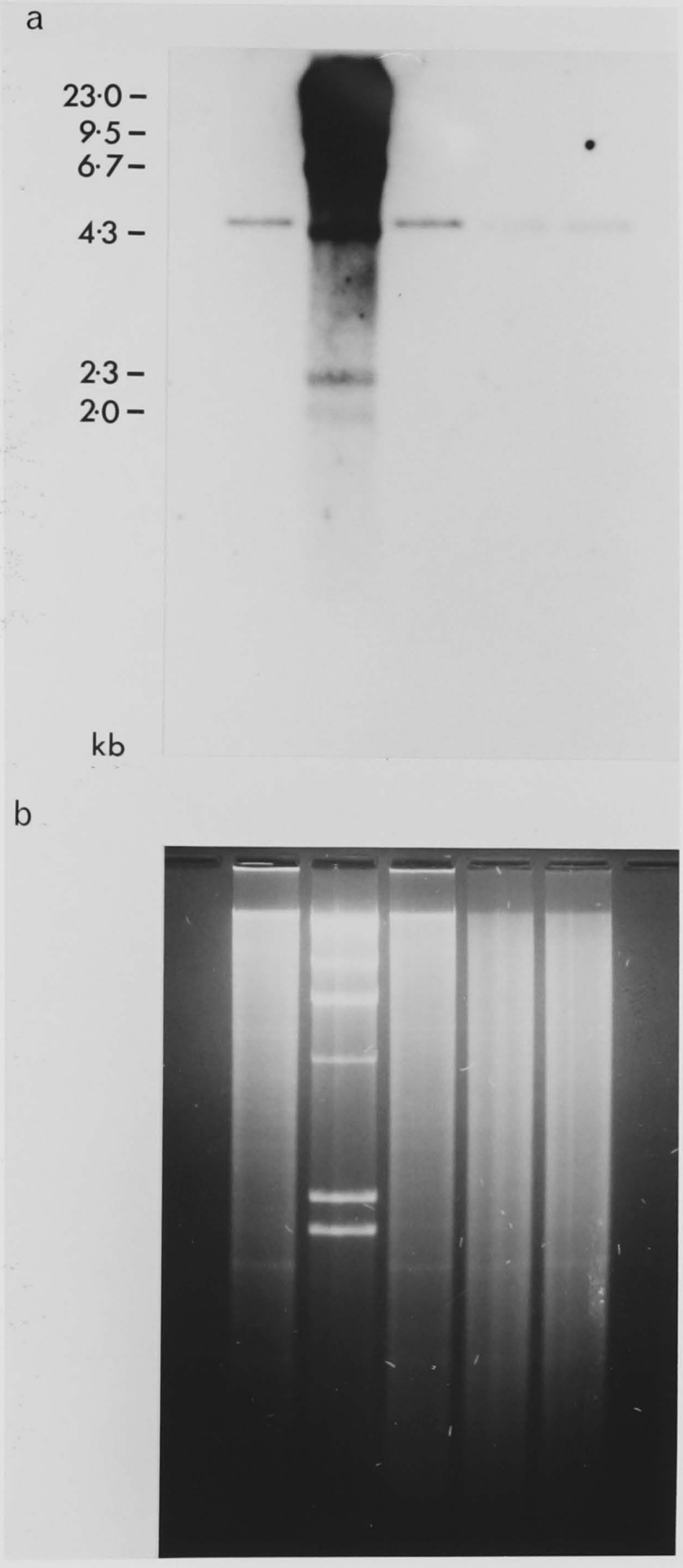
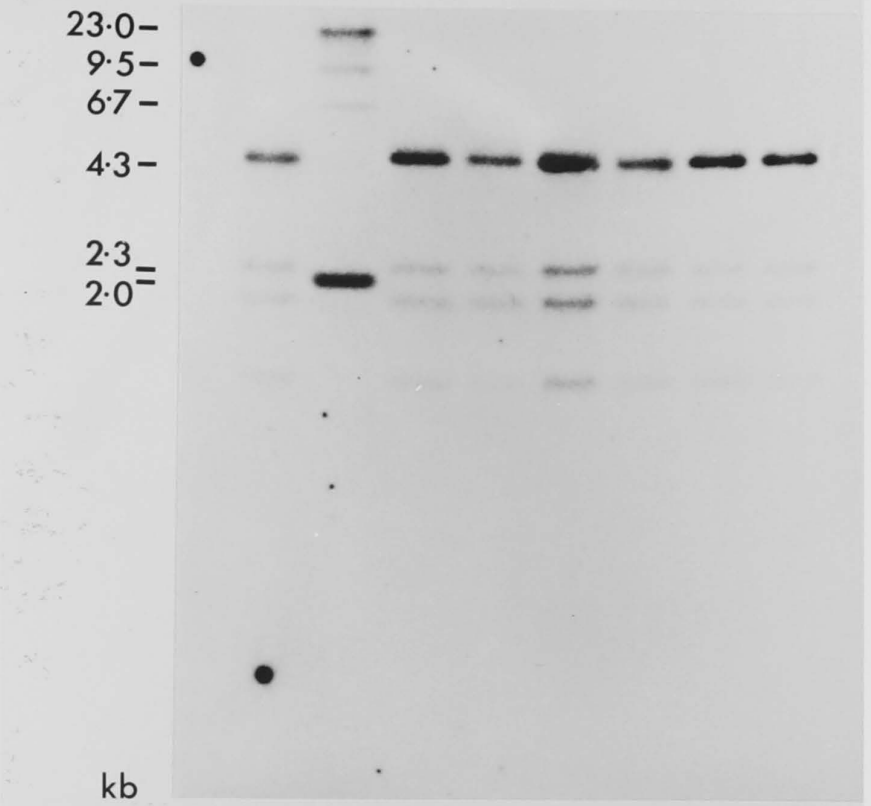

a
23·0 –
9·5 –
6·7 –
4·3 –
2·3 –
2·0 –
kb
b

Figure 6.3. Southern transfer of *Eco*RI-digested adult genomic DNA probed with sAF2 DNA (a) and the digested DNA prior to transfer as visualised by ethidium bromide staining (b). From left to right: Adh^{AC8}, λ*Hin*dIII, Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAC80}, Adh^{nAP81}, and Adh^{nAT33}.

a



b

CHAPTER SEVEN

INVESTIGATIONS OF THE TRANSCRIPTS PRODUCED BY THE *Adh* NULL ALLELES

7.1 Introduction

This Chapter describes investigations aimed at determining whether or not the Tasmanian *Adh* null alleles produce any mRNA. In addition, comparison of null alleles from different populations were made to provide evidence on possible heterogeneity in mRNAs produced by the separately extracted null alleles and this should help to resolve questions concerning their origin(s).

Southern blot analyses of the *Adh* null alleles isolated from Tasmania in 1983 and 1984 indicated that the *Adh* gene region was intact without any detectable insertions or deletions in the structural gene or the surrounding 7 kilo-base (kb) region. As the ELISA assays and Western blotting had failed to detect any ADH cross-reacting material these data, taken together, suggested that a defect in the transcription of the *Adh* gene or in the translation of its transcript was the cause of the loss of ADH activity. With respect to *Adh* transcripts three situations could be tested: (1) no mRNA is produced, implicating a defect in the control or initiation of transcription, (2) an mRNA indistinguishable from wild-type is produced, suggesting a mutation involving either early translation termination or the loss of the ADH antigenic determinant, and (3) an mRNA differing in size to the wild-type, is found, indicating mutations which interrupt transcription, RNA processing, and/or transcript translation. The aim of the experiments discussed in this Chapter was to classify the *Adh* null alleles on the basis of their mRNAs to provide some indication of the type(s) of mutation event responsible for the loss of ADH activity.

Benyajati *et al.* (1980) found that the adult *Adh* mRNA was about 1120 nucleotides long, was polyadenylated and accounted for about 1 to 2% of the translational activity found in adult flies. Later work

showed that the major second instar larval and adult transcripts of *Adh* differed in their 5' untranslated regions (Benyajati *et al.*, 1983; Henikoff, 1983). The 5' untranslated region of the 1100-base larval mRNA is colinear with the genomic sequence with a cap site 24 bp 3' to the proximal TATA box (Fig. 7.1). The major adult *Adh* transcript is an 1150-base mRNA which shares only 36 of its 123 5' untranslated bases with the larval mRNA; the remaining 87 are transcribed from a region located 25 bp 3' to the distal TATA box. A 654-base intron (intron 1) is removed to give the mature adult mRNA (Benyajati *et al.*, 1983). The position 36 bases 5' to the initiation codon (AUG) represents the boundary where the adult and larval transcripts differ; 3' to this position they are identical while 5' they contain different sequences (Fig. 7.1).

The series of experiments described in this Chapter only investigated *Adh* transcripts from adult flies. The Northern blotting technique described by Alwine, Kemp, and Stark (1977) and Thomas (1980) was used as it allowed the analysis of the mRNA both in terms of size and amount.

7.2 Materials and Methods

7.2.1 *Drosophila* strains

The Tasmanian *Adh* null alleles investigated (Adh^{nAC14}, Adh^{nAC22}, Adh^{nAC53}, Adh^{nAC80}, Adh^{nAP81}, and Adh^{nAT33}) and the control allele, Adh^{AC8}, have been described (Chapters Three and Four). Mass cultures of each homozygous strain were set up and checked for contamination by pentenol vapour screening. These cultures were maintained for two months at 25°C with periodic collections of adult male and female flies being frozen immediately in liquid nitrogen and stored at -70°C until

required. Another *Adh* null allele, Adh^{nAH9}, isolated from the Huonville II population in 1985 by Dr J.B. Gibson was also investigated and Ms A.V. Wilks provided collections of adult flies. Frozen Canton S male and female flies were provided by Dr G.L.G. Miklos.

7.2.2 DNA clones

The recombinant plasmids sACl and sAF2 used as DNA probes for *Adh* mRNA were as described in Chapter Six. Plasmid Hd-19, originally constructed by Dr S. Tobin was a gift from Dr J. Modolell to Dr G.L.G. Miklos. This pBR322 plasmid has an 1.8 kb insert (into the pBR322 *Hin*dIII site) which contains the coding region of the *D. melanogaster* actin 5C gene.

7.2.3 Extraction of RNA

Total cellular RNA was extracted from adult flies by the guanidine thiocyanate/caesium chloride procedure of Chirgwin, Przybyla, MacDonald, and Rutter (1979). About lgm of adult *Drosophila* were frozen in liquid nitrogen and ground to a fine powder with a pre-cooled mortar and pestle. To this powder, 8ml of extraction solution (4M guanidine thiocyanate, 0.05M sodium acetate, 1.0M β-mercaptoethanol mixed with 0.4g activated charcoal, filtered and 1% N-lauryl sarcosine and 5mM EDTA added) was added together with solid caesium chloride (4g). The mixture was heated to 60°C until all the solid material had dissolved and then quick-cooled on ice. The preparation was carefully layered over a 1.2ml caesium chloride cushion solution (5.7M caesium chloride, 0.01M Tris-HCl, pH7.5, 0.1M EDTA) and centrifuged for 18 to 22 hours at 35,000rpm (SW-65 rotor) at 15°C.

The RNA pellet was resuspended in T.E. buffer with 0.5% N-lauryl sarcosine. This suspension was heated at 80°C for 1 minute, quick-cooled on ice and treated with an equal volume of

phenol/chloroform/isoamyl alcohol (25:25:1). Aliquots of the RNA-containing solution were dispensed into microfuge tubes (Eppendorf) and precipitated overnight with ethanol at -70°C (400ul RNA solution, 40ul of 3M sodium acetate, 1ml of ethanol). RNA from one tube was recovered by centrifugation at 15,000rpm (Eppendorf 5415S), dried under vacuum, resuspended in 50ul of T.E. buffer, and the RNA concentration determined by OD readings at 260nm. The remaining tubes of RNA were stored at -20°C until required.

Glassware used for RNA extractions and for subsequent experiments investigating mRNAs was baked at 200°C for at least 6 hours to destroy ribonuclease activity. Disposable Pipetman tips and Eppendorf microfuge tubes were autoclaved as were all solutions and double distilled water containing diethyl pyrocarbonate.

7.2.4 Preparation of RNA samples

RNA was recovered from the microfuge tubes as described above and resuspended in water at 37°C for 5 minutes to give a final concentration of 30ug/5ul. RNA was prepared for electrophoresis by the glyoxal and dimethyl sulfoxide (DMSO) denaturation method of McMaster and Carmichael (1977). Mixed bed resin (Bio-Rad) columns were used to deionise glyoxal and DMSO just prior to sample preparation. The denaturing reaction mixture contained 50% deionised DMSO, 6% deionised glyoxal, 10mM NaH_2PO_4/Na_2HPO_4 buffer, pH7.0 and 5ul of RNA solution. This mixture was incubated at 50°C for 1 hour, after which it was quick-cooled on ice and 2ul of sample dye (50% glycerol, 2.5mM phosphate buffer, pH7.0, 0.09% bromophenol blue) was added and samples were used immediately for electrophoresis. DNA size markers, lambda (c1857) *Hin*dIII, were denatured as described before electrophoresis.

7.2.5 Electrophoresis and Northern blotting

Horizontal gel electrophoresis with 1% or 1.5% agarose gels was as described by McMaster and Carmichael (1977). Gels were prepared in electrophoresis buffer (10mM sodium phosphate buffer, pH7.0) and electrophoresis was for 14 to 16 hours at 30V (20-25mA) with buffer recirculation. After electrophoresis, denatured RNA was transferred to nitrocellulose by overnight blotting in 20 x SSC according to the method of Thomas (1980). The nitrocellulose filters were then baked under vacuum at 80°C for 2 hours and stored until required.

7.2.6 Hybridisation of Northern blots

Hybridisation of filters with radiolabelled DNA was modified from Thomas (1980). Nick translated DNA hybridisation probes were made by incubating plasmid DNA (20ul of DNA-containing solution) with a mixture of deoxyribonucleotides (dATP, dGTP, and dTTP at about 2mM) in nick translation buffer (5ul of 0.5M Tris-HCl, pH7.2, 0.1M $MgSO_4$, 500ug/ml BSA and 1mM dithiothreitol) and water (15ul). To this reaction mixture DNAase-1 (2ul of 0.1M, Sigma), 1ul of DNA polymerase 1 (Bresa at 10 ug/ml) and 3ul of [α-^{32}P]-dCTP (30uCi) were added and the mixture was then incubated at 16°C for 2 hours. The reaction was stopped by placing the mixture at 70°C for 10 minutes before passing it through a Sephadex-G50 column as previously described.

Prehybridisation of filters was in 5 x SSC with 50% freshly deionised formamide, 10 x Denhardt's solution, 50mM sodium phosphate buffer, 0.1% sodium dodecyl sulphate (SDS), 0.1% tetrasodium pyrophosphate and heat-denatured herring sperm DNA (23ug/ml). Prehybridisation was at 42°C for 1 to 3 hours after which the heat-denatured radioactive probes were added and hybridisation allowed to proceed overnight for 15 to 18 hours at 42°C. Next day the filters were

rinsed in 2 x SSC, washed with 2 x SSC containing 0.1% SDS and 0.1% tetrasodium pyrophosphate at 65°C for 30 minutes (2 x) followed by a single wash for 30 minutes at 65°C in 0.2 x SSC with 0.1% SDS and sodium pyrophosphate. The filters were air dried for 20 to 30 minutes before autoradiography as described for Southern hybridisations.

If filters were to be rehybridized with a different DNA probe they were washed in 0.05 x wash buffer (50mM Tris-HCl, pH8.0, 2mM EDTA, 0.5% sodium pyrophosphate, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinyl-pyrrolidone) at 65°C for 2 hours and exposed to X-ray film to ensure all the probe had been removed. Prehybridisation and hybridisation proceeded as described. Hybridisation intensities of transcript bands were measured with a Quick Scan integrating densitometer (Helena Laboratories).

7.3 Results

Adh and *act5C* transcription patterns for Adh^{AC8} and five of the 1983 null alleles are shown in Figure 7.2. *Adh* transcripts were produced by all five null alleles, but in each the transcripts were present in low amounts compared to Adh^{AC8}. In addition, the more cathodal position of the null transcripts indicated a longer than wild-type mRNA (Fig. 7.2a). Two *act5C* transcripts of higher molecular weight than the *Adh* mRNA were detected in both the control and the null strains (Fig. 7.2b). Total RNA loaded in each lane appeared to be equivalent as judged by the relative hybridisation intensities of both *act5C* transcripts for each strain. Adh^{nAC53} and Adh^{nAH9} transcription patterns were determined in a separate experiment. Both had *Adh* transcripts of longer length than wild-type and at reduced levels similar to those observed for the other five null alleles (Fig. 7.3a).

Hybridisation of the Adh^{nAC53} band appeared more intense, and was due to more total RNA being loaded as indicated by the actin controls (Fig. 7.3b). In another experiment, Adh^{nAC14} and Adh^{nAC22} appeared to be accumulating greater amounts of an *Adh* mRNA precursor than the Canton-S control (Fig. 7.4). Ms A.V. Wilks (*personal communication*) has demonstrated a similar precursor accumulation in the *Adh* null alleles isolated from the Tasmanian populations in 1984 and 1985 and has estimated the size of this precursor to be about 1800-2000 bases.

To quantify the intensity of hybridisation and hence the relative amounts of mRNA, densitometer scans of each transcript were made. Table 7.1 lists the amounts of *Adh* mRNA and *act5C* mRNA present in Adh^{AC8} and in the seven null strains based on three separate Northern experiments. Also listed are corrected *Adh* mRNA amounts obtained by dividing each *Adh* mRNA amount by the values for the two actin transcripts. (The faster migrating *act5C* transcript is designated 2). This calculation allows for any differences in the amounts of total RNA loaded for each strain.

Actin mRNA amounts in the same strain were often variable but, overall, comparable amounts were detected both in the Adh^{AC8} control and the null strains. On average, uncorrected and corrected *Adh* mRNA amounts present in the seven null strains (which did not differ in amounts of *Adh* mRNA) were about 10% of that found for Adh^{AC8}. Although samples sizes are low, the standard errors suggest that the differences are significant (Table 7.1). In another experiment, mRNA amounts of two null strains, Adh^{nAC14} and Adh^{nAC22}, were compared to transcript levels found in Canton-S flies (Table 7.2). Differing levels of mRNA were detected for the two actin transcripts in Adh^{nAC22} and Canton-S and this affected the corrected *Adh* mRNA values. However, comparing uncorrected

amounts both Adh^{nAC14} and Adh^{nAC22} had about 10% of the *Adh* mRNA found in the Canton-S control.

Size estimates of the *Adh* and actin transcripts for Adh^{AC8} and five of the 1983 null alleles were each made at two different agarose concentrations (Table 7.3). The two transcripts produced by *act5c* migrated to positions corresponding to sizes of about 1600 bases (1567-1748) and 1400 bases (1369-1540). On average, the five 1983 null alleles produced an *Adh* mRNA which was about 100 bases longer than the wild-type control.

The position of *Adh* mRNA bands observed for Adh^{nAC14}, Adh^{nAH9} and Adh^{nAC53} (Fig. 7.3) are consistent with these alleles producing transcripts of similar lengths.

7.4 Discussion

The *Adh* mRNAs produced by the six *Adh* null alleles isolated from Tasmania in 1983 and by one null allele isolated in 1985 were characterised by Northern transfer hybridisation experiments. The mRNAs produced by the Tasmanian *Adh* null alleles were found to differ from the *Adh* transcript of a standard Adh^{S} allele both in size and amount.

Estimates based on hybridisation intensities indicated that null allele homozygotes had *Adh* mRNA levels about a tenth of that found in wild-type flies. Nevertheless, there are a number of problems associated with quantifying mRNA amounts from Northern blot autoradiographs. The accuracy of the densitometer scans is dependent on the gel loadings, homogeneous transfer of material from the filter, and levels of background hybridisation. All are difficult to control in hybridisation experiments and any variation in these conditions could result in quite different scan profiles. Visual inspection of the

autoradiographs from which scans were made indicated that uneven band intensity and some background hybridisation may, in part, account for the variation observed.

An actin gene, *act5C*, was used to standardise comparisons between null and control strains. There are six *D. melanogaster* actin genes which form a highly conserved family, encoding similar, but not identical proteins (Fyrberg, Bond, Hershey, Mixter, and Davidson, 1981). The expression of the six genes varies independently in a stage-specific manner; *act5C* produces three transcripts, one of which (size 1.95 kb) is relatively abundant in adult flies, while the remaining two (2.2 and 1.7 kb) are present in low amounts (Fyrberg, Mahaffey, Bond, and Davidson, 1983). In these experiments only the 1.95 kb and the 1.7 kb transcripts were detected in adult material and usually both were present in about equal amounts. As the relationship between the three transcripts of the *act5C* gene has not been determined, both of them were used to correct for any differences in total RNA amounts.

The chief finding was that all of the *Adh* null alleles gave transcripts which were consistently larger than the wild-type by an amount ranging from 71 to 119 bases. Size estimates for both *act5C* transcripts were smaller than those reported by Fyrberg *et al.* (1983), but the size estimates of 963 bases and 1105 bases for the mRNA produced by Adh^{AC8} were close to the reported value of 1150 for the wild-type transcript.

In all the nulls investigated, the lack of ADH protein must be the result of mutations affecting mRNA processing or mRNA stability. Some general points, however, can be made about the kind of mutations responsible. First, it is unlikely that the longer *Adh* mRNA observed in the Tasmanian nulls is due to a duplication as found in Adh^{nLA248} by

Chia *et al.* (1985) since structural alterations have not been detected in the Tasmanian nulls, whose Southern blot restriction patterns appear to be indistinguishable from the wild-type control line. Adh^{nLA248} is an X-ray induced *Adh* null with no detectable ADH protein, however, it produces a transcript which is about 200 bases longer than normal and has a corresponding 250 bp increase in the region covered by the sAC1 clone, which is detectable by Southern blot analysis (Chia *et al.* 1985; Kelley *et al.*, 1985).

The Tasmanian null alleles might be caused by a mutation affecting the processing of either introns 2 or 3 and this would be consistent both with the genomic DNA data and the observed mRNA transcript length. A number of investigations have linked abnormal intron splicing to instability of mRNAs eg., simian virus 40 mRNAs and human haemoglobin mRNAs (Gruss, Lai, Dhar, and Khoury, 1979; Orkin, Goff, and Hechtman, 1981; Fukumaki, Ghosh, Benz, Reddy, Lebowitz, Forget, and Weissman, 1982). Similar findings have been reported by Benyajati *et al.* (1982) for two formaldehyde-induced *Adh* null alleles Adh^{fn4} and Adh^{fn6}. Both are homozygous viable, lack detectable ADH cross-reacting material and show no changes in either restriction sites or sizes of DNA fragments in Southern blot experiments. No mature adult mRNA was detected, but the mutants produced low amounts (5 to 10% of wild-type) of a 1900-base *Adh* transcript. From DNA sequence analysis, Benyajati *et al.* (1982) infer that the defect in RNA processing is a consequence of two small deletions in intron 2; a 17 bp deletion which removes the 3' splice site sequences in Adh^{fn4} and a 6 bp deletion which removes the 5' splice site sequences in Adh^{fn6}. No other changes were detected and Benyajati *et al.* (1982) suggest that splicing of the *Adh* mRNA may proceed in a specific order such that mutations in the 65 bp intron 2 affect the

removal of the 654 bp intron 1. Order specific intron processing has been observed in rabbit beta-globin, *Xenopus* vitellogenin, and adenovirus 2 transcripts (Grosveld, Koster, and Flavell, 1981; Ryffel, Wyler, Muellener, and Weber, 1980; Berget and Sharp, 1979) and Sharp (1981) has suggested this results from the varying efficiency with which splice sites are recognised.

In the Tasmanian *Adh* nulls an *Adh* mRNA precursor of about 1800 to 2000 bases accumulated at levels greater than found in the wild-type control, although the dominant transcript detected was about 100 bases longer than normal. A mutation in the 5' or 3' splice junction of either intron 1 or intron 2 may prevent their removal and reduce the overall efficiency of mRNA processing, leading to the accumulation of an unprocessed transcript of 1801 bases containing all three introns. The observed increase of 100 bases in the null allele transcripts is larger than either intron 2 (65 bp) or intron 3 (70 bp). However, this size difference is unlikely to be significant given the variation observed in size estimates from different Northern experiments.

Benyajati *et al.* (1982) were unable to determine if the lower levels of mRNA in Adh^{fn4} and Adh^{fn6} were due to reduced transcriptional efficiency or to a less stable mRNA. Similar levels of *Adh* mRNA were observed in the Tasmanian nulls and these could also be due to reduced rates of transcription and/or a reduced mRNA stability associated with splice site mutations.

To conclude, this preliminary investigation has shown that the mutations responsible for the loss of ADH activity in the Tasmanian *Adh* nulls examined affects the production and/or the processing of the mRNA encoded by these alleles. It is unlikely that the mutation(s) involves major insertions or deletions in the coding or noncoding regions,

although smaller alterations, undetectable by Southern analysis cannot be ruled out. Finally, the lack of heterogeneity in the null allele transcripts, within and between populations, supports the suggestion that these separately isolated alleles are copies of the same mutation. It remains possible, however, that there are several different mutations, as for Adh^{fn4} and Adh^{fn6}, which each result in very similar sized mRNAs. Resolution of the origin of these mutants will be aided by information on the exact defect(s), and for this DNA sequence studies are required.

Table 7.1. *Adh* and actin *act5C* transcript amounts (mean with standard error in parentheses) as determined for Adh^{AC8} and the seven Tasmanian *Adh* null alleles.

Allele	n	*Adh* mRNA	Actin mRNA		*Adh* mRNA/Actin mRNA	
			1	2	1	2
AC8	3	191.05 (9.36)	36.12 (7.40)	40.06 (7.79)	5.66 (0.95)	5.18 (1.07)
AC14	3	13.89 (2.26)	51.74 (10.41)	55.93 (28.52)	0.30 (0.09)	0.43 (0.17)
AC22	2	11.61 (0.06)	88.95 (63.17)	35.93 (6.31)	0.26 (0.19)	0.33 (0.06)
AC53	1	36.56	118.64	65.03	0.31	0.56
AC80	2	20.56 (4.81)	34.64 (0.12)	25.37 (7.84)	0.59 (0.14)	0.83 (0.07)
AP81	2	18.43 (0.71)	25.11 (1.00)	30.60 (2.54)	0.74 (0.06)	0.60 (0.03)
AT33	2	18.57 (3.55)	46.02 (7.53)	44.85 (20.62)	0.43 (0.15)	0.48 (0.14)
AH9	1	18.36	57.72	18.83	0.32	0.98

Table 7.2. *Adh* and actin *act5C* transcript amounts as determined by densitometer scans for Canton S, Adh^{nAC14}, and Adh^{nAC22} from one Northern experiment.

Allele	*Adh* mRNA	Actin mRNA		*Adh* mRNA/Actin mRNA	
		1	2	1	2
S	344.70	46.23	122.41	7.46	2.82
AC14	34.47	77.29	55.58	0.45	0.62
AC22	28.39	69.05	33.74	0.41	0.84

Table 7.3. *Adh* and actin *act5C* transcript sizes as estimated from two separate Northern transfers using 1.0% and 1.5% gel agarose concentrations.

Allele	*Adh* mRNA		Actin mRNA			
			1		2	
	1.0%	1.5%	1.0%	1.5%	1.0%	1.5%
AC8	963	1105	1567	1726	1369	1520
AC14	1082	1191	1567	1704	1369	1501
AC22	1064	1176	1621	1704	1393	1501
AC80	1082	1191	1567	1704	1369	1501
AP81	1064	1191	1567	1704	1369	1501
AT33	1064	1206	1567	1748	1369	1540

Figure 7.1. Diagrammatic representation of the *Adh* gene and the alternative transcription and splicing patterns for the predominant second instar larval and adult transcripts. Exons (ex) are shown as boxes and introns (in) as lines; the untranslated 5' regions of exons are shown hatched while the 36-base region common to both transcripts is crosshatched. The sizes of the coding and noncoding regions are shown as base-pairs. (Modified from Chia *et al.*, 1985).

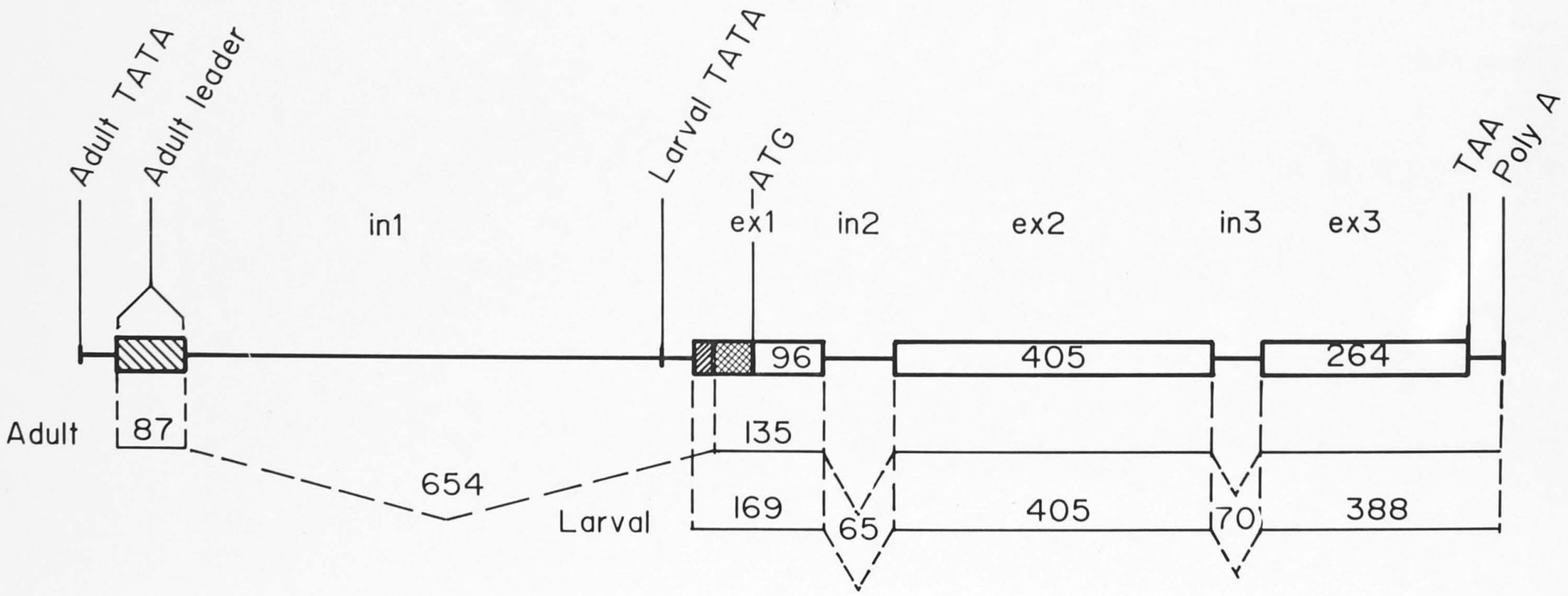

Adult TATA
Adult leader
Larval TATA
ATG
TAA
Poly A
in1
ex1
in2
ex2
in3
ex3
96
405
264
Adult
87
654
135
Larval
169
65
405
70
388

Figure 7.2. Northern transfer of total adult mRNA probed with sAC1 DNA (a) and later reprobed with Hd-19 DNA (b). Gel agarose concentration was 1.5%. From left to right: Adh^{AC8}, λ*Hin*dIII, Adh^{nAC14}, Adh^{nAC22}, blank, Adh^{nAC80}, Adh^{nAP81}, Adh^{nAT33}.

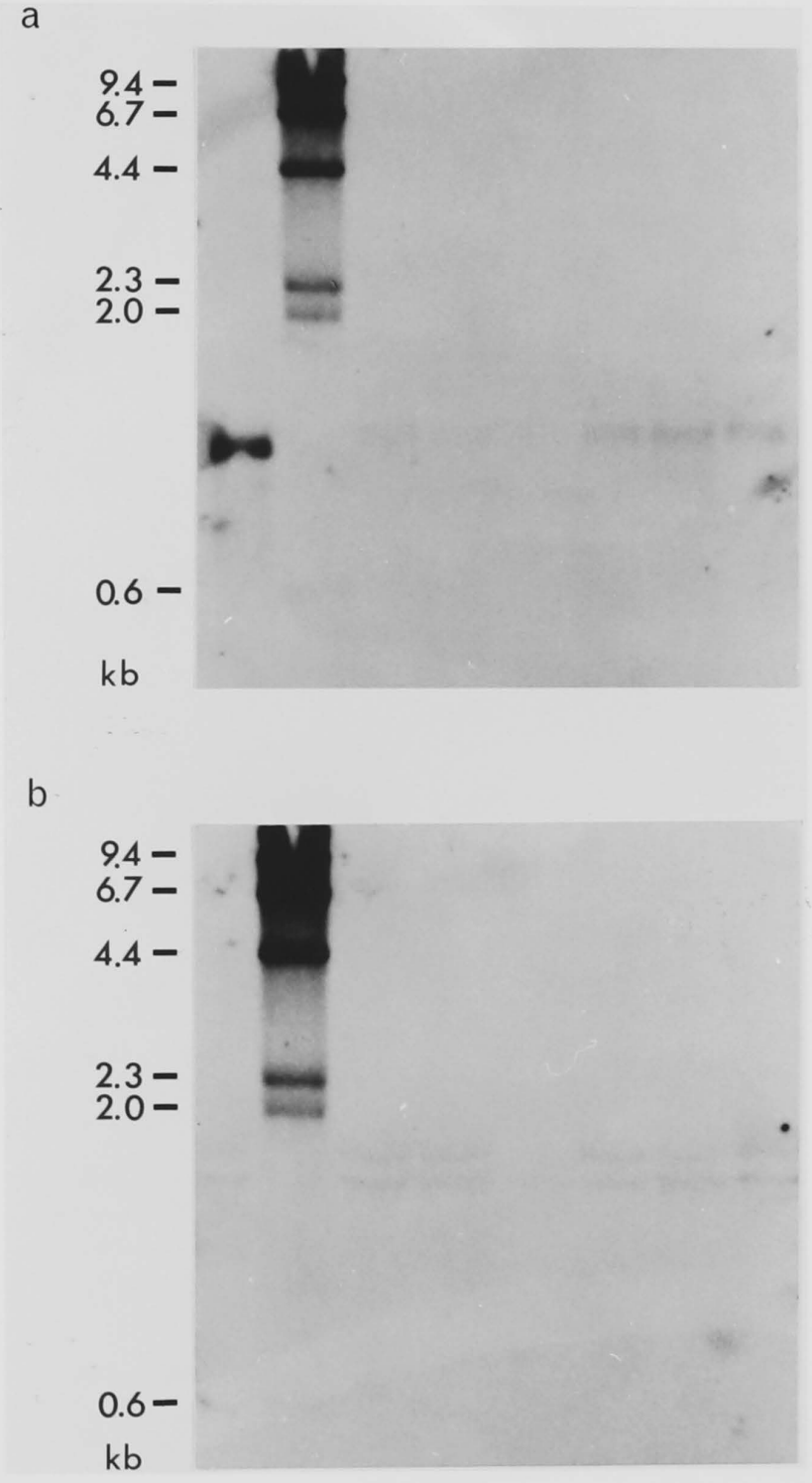

a
9.4
6.7
4.4
2.3
2.0
0.6
kb
b
9.4
6.7
4.4
2.3
2.0
0.6
kb

Figure 7.3. Northern transfer of total adult mRNA probed with sAF2 DNA (a) and later reprobed with Hd-19 DNA (b). Agarose concentration was 1.0%. From left to right: Adh^{AC8}, blank, Adh^{nAH9}, Adh^{nAC14}, Adh^{nAC53}.

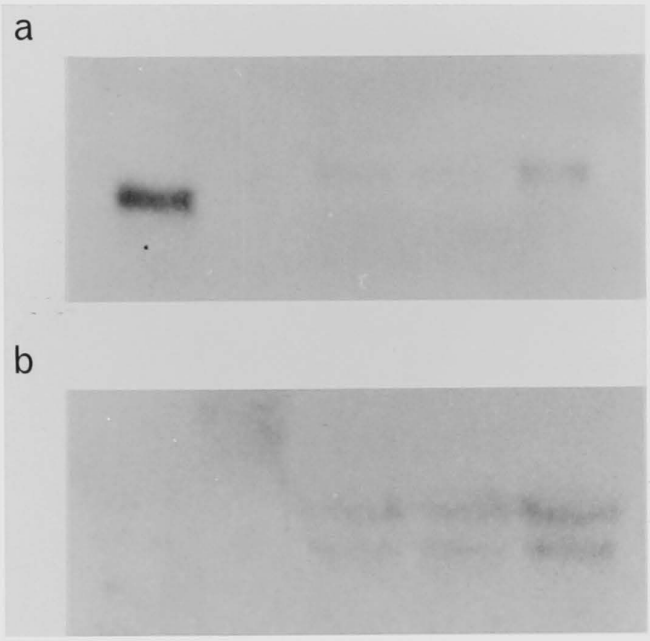
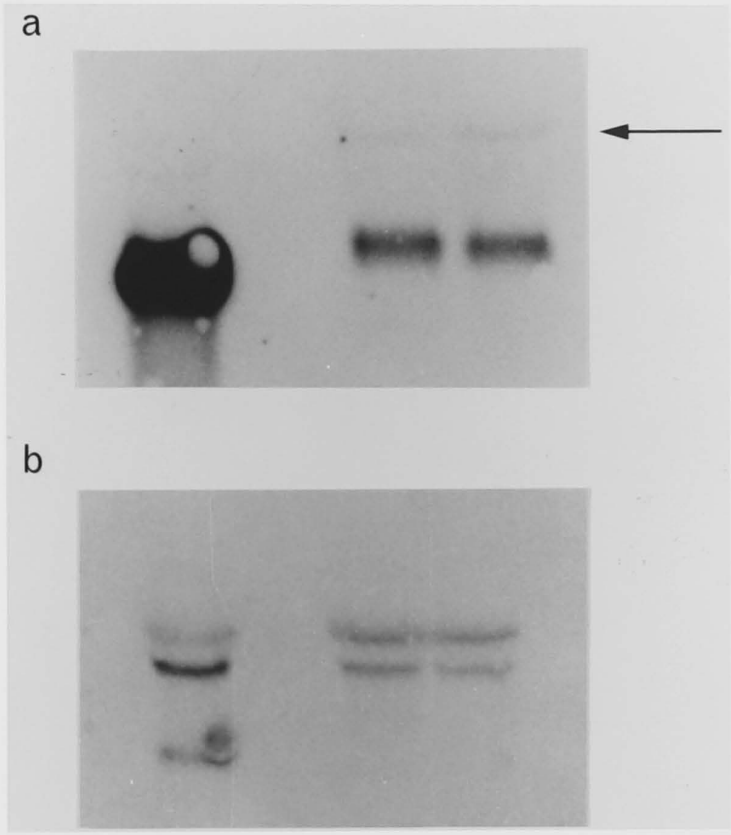

a
b

Figure 7.4. Northern transfer of total mRNA probed with sACl DNA (a) and later with Hd-19 DNA (b). Agarose gel concentration was 1.0%. The arrow indicates the accumulated precursor *Adh* mRNA. From left to right: Canton S, blank, Adh^{nAC14}, Adh^{nAC22}.


a
b

CHAPTER EIGHT

GENERAL DISCUSSION

The discovery of *Adh* null alleles in natural populations of *D. melanogaster* at, or close to, polymorphic frequencies was unexpected given the previous evidence about the occurrence and frequency of null alleles at allozyme loci in *Drosophila*. The *Adh* locus is not normally duplicated in *D. melanogaster*, and previously only a single naturally occurring putative null allele had been claimed in this organism but this was later shown to produce a low level of active protein. Also, ADH is found at high levels in third instar larvae and adults, and is thought to have a primary function in ethanol metabolism. Thus, the occurrence of null alleles at the *Adh* locus in the Tasmanian *D. melanogaster* populations has raised a number of questions concerning the origin and maintenance of these alleles in the populations sampled, and the ecological relevance of the ADH system as a whole.

In attempting to answer these questions it is important to first decide: (a) how many different *Adh* null alleles were present in the Tasmanian populations? and (b) how does the frequency of *Adh* nulls compare with those previously reported for other natural populations?

A large part of the research described in this thesis has been concerned with the first question, that is, the determination of how many different *Adh* null alleles were present. The approach adopted was to first biochemically characterise the null alleles to ascertain if any had residual activity or produced ADH protein. Later, the DNA and RNA studies were carried out to detect any molecular heterogeneity between the null alleles and identify the class of mutation responsible. Ultimately, DNA sequencing of the mutant alleles will be required to fully answer the question.

Nine null alleles were isolated from the Tasmanian populations in 1983 and the characterisation of these alleles has formed the bulk of the research described in this thesis. In addition, *Adh* null alleles isolated by Dr J.B. Gibson from the 1984 and 1985 Tasmanian population collections were also investigated and Ms A.V. Wilks provided information from her molecular analyses of these alleles. Although in 1983 not all the *Adh* null alleles detected were isolated, in 1984 and 1985 every null allele found was successfully extracted. Therefore, the sample is believed to be representative of the *Adh* null alleles present in each population.

It is important to remember that all of the null alleles were derived from four geographically separate populations, despite the tendency to refer to them as 'Tasmanian null alleles'. Cygnet and Huonville are about 10km apart in southern Tasmania, some 300km from the Pipers Brook and Tamar Valley wineries (30km apart). Central Tasmania lacks any major breeding resources for *Drosophila* and surveys carried out in 1984 failed to trap any *D. melanogaster* in this region even at domestic rubbish dumps. Therefore, it is possible that these populations are partially isolated from one another, although it is doubtful if this isolation is complete given the transport of fruits (particularly apples from the south) and other material throughout Tasmania by man.

Several models can be proposed to explain the occurrence of *Adh* null alleles at these four locations. A single mutational event may have given rise to a null allele which later spread to the other populations. Alternatively, a series of independent but similar mutations might have occurred in each of the four populations and produced a number of different null alleles or a mutational 'hot-spot'

within the *Adh* gene could have resulted in identical null mutants occurring in all four populations.

Is there any evidence supporting the hypothesis of an independent origin for the *Adh* nulls detected in each of the four Tasmanian populations? Table 8.1 summarises my research on the 1983 *Adh* null alleles and the research of Dr J.B. Gibson and Ms A.V. Wilks on the null alleles isolated from Huonville II and Tamar Valley in 1984 and 1985. Apart from one Huonville 1985 allele and three from Cygnet in 1983, all the null alleles were homozygous viable. The inviability of Adh^{nAC41}, Adh^{nAC60}, and Adh^{nAC62} was found to be due to a lethal which was independent of the *Adh* gene. Three other strains, all from the Huonville II population were found to be homozygous sterile. However, the 19 homozygous viable null alleles were found to be identical for every character investigated (Table 8.1). As far as could be determined within the resolution of the various techniques, no allele had a major alteration or rearrangement in the *Adh* gene region, while all contained low amounts of a longer than normal *Adh* mRNA. All lacked ADH activity and, in the alleles tested, no ADH cross-reacting material was detected.

Given this apparent homogeneity in properties it does not appear as if these alleles are the result of different mutational events, at least not at this level of resolution. If different mutations had occurred, then they have all resulted in identical biochemical and molecular phenotypes. The molecular analyses suggest that the mutation(s) affects *Adh* mRNA function, and the range of mutation types which can account both for the reduced mRNA amounts and the altered mRNA length is limited. Taken together, the available data strongly supports the suggestion that these null alleles are either multiple copies of the same mutant, or the products of mutation at a hot-spot which has produced identical null alleles in the Tasmanian populations.

There is some indirect evidence which would argue against a hot-spot induced, independent origin of nulls in the four populations. Hot-spots of mutational activity have long been associated with mobile chromosome elements and hybrid dysgenesis (Green, 1977; Woodruff *et al.*, 1983). Generation of the *Adh* null alleles by these or similar mechanisms would appear unlikely given the evidence indicating a low level of such mutator activity in the Tasmanian populations and the reported difficulty in inducing P-element insertions in the *Adh* gene (see Woodruff *et al.*, 1983).

In addition, endonuclease restriction maps of the 11.8kb *Adh* region covered by Goldberg's sAF2 clone have been prepared by Ms A.V. Wilks for the null alleles isolated in 1984 and 1985 using eight restriction endonucleases. All these nulls show identical Southern blot patterns, identical to that found for the Adh^S control allele (Adh^{AC8}), and conforming to the restriction map described by Goldberg (1980) for the sAF2 clone. This is despite the existence of considerable restriction site variation in this region among chromosomes with active *Adh* alleles extracted from the same Tasmanian populations (Dr J.B. Gibson, Ms A.V. Wilks, and Mr Chengshan Jiang, *personal communication*). If a hot-spot does exist in the *Adh* gene, then it should be present in alleles borne on a range of chromosome types, i.e., with respect to restriction site haplotypes. The association of the 1984 and 1985 null alleles from Tamar Valley and Huonville II (300km apart) with one particular haplotype is further evidence supporting a single origin for these alleles. Any suggestion that a particular *Adh* haplotype is more prone to mutation shifts the argument from the geographical distribution of null alleles to the distribution and origin of a mutable haplotype.

Final resolution of the origin of these null alleles awaits DNA sequence studies on samples of null alleles from each of the studied populations, but the implications of a single origin model will be further discussed below. For the present, I wish to concentrate on the probable cause(s) for the loss of ADH activity in the null alleles.

Any explanation concerning the nature and location of the mutation(s) involved has to account for: (1) the lack of detectable ADH cross-reacting material, (2) the apparent structural integrity of the *Adh* gene and the surrounding 7kb region, and (3) the presence in adults of an *Adh* mRNA which is about 100 bases longer than wild-type and detected in greatly reduced amounts (10%).

It is unlikely that a mutation in a regulatory locus or a controlling element of the *Adh* gene is responsible for the observed phenotypes given the altered size of the mRNA. *Cis*-acting regulatory elements have been demonstrated for *Adh* in the Hawaiian picture-winged *Drosophila,* in *D. mulleri*, and in *D. melanogaster* (Rabinow and Dickinson, 1986; Fischer and Maniatis, 1986; Goldberg *et al.*, 1983). Goldberg *et al.* (1983) found that the 11.8kb chromosomal region of the sAF2 clone contained all the *cis*-acting sequences necessary for correct *Adh* expression in *D. melanogaster*. Later, it was determined that there are at least two physically-separable elements necessary for expression of the proximal (larval) *Adh* promoter. Sequences necessary for the temporal and tissue-specific expression of transcripts initiated from the proximal promoter are within 400bp of the promoter, but an element required for normal levels of transcription is located at least 2kb upstream (Fischer and Maniatis, 1986). Although there is no structural disruption to the 5' region of the null alleles, as covered by the sAF2 clone, smaller undetectable deletions or insertions may have occurred in

cis-acting elements thereby affecting levels of transcription. However, such a mutation would not account for the increased mRNA length and for the present it is best to avoid any explanation requiring two or more separate mutations. Nevertheless, once totally or partially 'silenced' by a mutation, an *Adh* allele could accumulate other mutations in a manner analogous to intervening DNA sequences or duplicated gene loci (Li, 1980).

It is of interest to note that few allozyme null alleles have been found to be caused by regulatory defects. Isocitrate dehydrogenase (Idh^{GB1}) and malic enzyme (Me^{NC1}) null alleles isolated by Langley *et al.* (1981) from two natural populations of *D. melanogaster* have been cited as possible regulatory mutants. Subsequent investigation of both alleles revealed them to be low activity variants, with reduced amounts of cross-reacting material in proportion to the observed activity. Williamson (1982) has suggested that Me^{NC1} represents a mutation in a regulatory locus controlling the levels of malic enzyme, as analyses of the protein have indicated no differences in electrophoretic mobility, molecular weight, substrate kinetics, or temperature optima when compared to wild-type. A similar situation was found for Idh^{GB1} which is believed to result from a *cis*-acting control mutation for one of at least two loci involved in IDH production (Bentley *et al.*, 1983). Schwartz and Sofer (1976) in a survey to detect possible *Adh* control mutations have analysed 16 ethyl methanesulfonate-induced *Adh* null alleles and identified 11 as being structural defects. Similarly Gelbart, McCarron, Pandey, and Chovnick (1974) found that most of their null rosy mutants were contained within the structural gene for xanthine dehydrogenase.

There are several types of mutation which could occur in the structural region of the *Adh* gene and lead to loss of gene expression via an altered mRNA. Major structural alterations or rearrangements such as deletions, insertions, inversions, or duplications of DNA sequences as reported for some artificially-induced nulls (O'Donnell *et al.*, 1977; Chia *et al.*, 1985; Kelley *et al.*, 1985) appear unlikely in the light of the Southern analyses. Alternatively, and more likely, are base substitutions, or minor deletions or insertions which disrupt or alter normal mRNA processing.

The lack of CRM in sensitive immunological tests indicates that either the mRNA cannot be translated or is only partially translated into small undetectable polypeptides, which are unstable and subject to rapid degradation. This might suggest that the extra 100 bases observed in the null *Adh* mRNAs is present in the central region of the transcript and not derived from the 3' untranslated region contiguous with the third exon. An attractive hypothesis therefore is that the mutant *Adh* mRNAs observed in the Tasmanian nulls result from a defect in the splicing of either intron-2 or intron-3 from the *Adh* mRNA precursor. This partially processed mRNA may be unstable, or alternatively the rates of RNA transcription and/or RNA processing may be reduced. The presence of an *Adh* mRNA precursor of about 1800 to 2000 bases at greater levels than found in the control strain points to a lower rate of mRNA processing, although no conclusion can be made concerning transcription rates; if the rate of precursor processing is sufficiently low, then low or high rates of transcription could still result in the observed precursor accumulation.

The accumulation of the precursor is consistent with a suggestion by Benyajati *et al.* (1982) that splicing of *Adh* mRNA may proceed in a

specific order such that mutations in either intron-2 or intron-3 may reduce the overall efficiency of processing. The Tasmanian *Adh* null alleles have similar protein, Southern DNA, and Northern RNA phenotypes to those reported by Benyajati *et al.* (1982) for two formaldehyde-induced *Adh* nulls. DNA sequence analyses of the latter found a small deletion in each, located at the 5' and 3' splice site junctions of intron-2. Accordingly, future work on the Tasmanian nulls will concentrate on cloning and sequencing the regions containing the splice site junctions of the two small *Adh* introns. As a number of different mutations could disrupt intron-exon boundaries (e.g., base substitutions, insertions, or deletions), sequence studies will provide crucial information relevant to the identity and origin of the Tasmanian null alleles.

The second major area of interest is how the observed frequencies of *Adh* nulls in Tasmania compares to those reported for other natural populations. As already mentioned, the only systematic studies of null allele frequencies in natural populations have been those of Voelker *et al.* (1980a), Langley *et al.* (1981), Mohrenweiser (1981), and Allendorf *et al.* (1982). Null allele frequencies for the allozyme loci screened in these four surveys were compatible, despite the diversity of the organisms studied, with mean null allele frequencies ranging from 0.23% to 0.31%. The range and distribution of frequencies observed were deemed to be consistent with a balance between mutation to, and selection against, the null allele.

The average frequency of *Adh* null alleles in the 1983 Tasmanian collections was 1.6%. The inclusion of the Coffs Harbour data reduces this average to 1.3%, but this still remains 14 times the value reported by Langley *et al.* (1981) for their Raleigh and London *D. melanogaster*

samples. They had detected only one *Adh* null allele out of 1170 alleles screened (0.09%). In 1984, *Adh* null allele frequencies were lower than in the previous year, but the average null frequency in Tasmania remained over seven times higher than the Raleigh and London value. Comparison of the Tasmanian 1983 and 1984 frequencies with the mean value of Langley *et al.* (1981) for 20 autosomal loci (0.24%) still gives the impression of an elevated frequency of *Adh* nulls in Tasmania.

In fact, the difference between these average frequencies is even larger as many of the null alleles identified by Langley *et al.* (1981) were, on subsequent investigation, shown to be low activity variants. Table 8.2 lists the original data for the 20 autosomal loci from Langley *et al.* (1981) together with my corrections based on published and unpublished reports. Most null alleles were found for sn-glycerol-3-phosphate dehydrogenase (*Gpdh*; abbreviations as listed in Chapter One) and aldehyde oxidase, but Burkhart *et al.* (1984) and Dr J.B. Gibson (*personal communication*) have found that all these alleles encode for an enzyme of residual activity (e.g., between 10 and 40% of wild-type for the *Gpdh* alleles). Similarly Williamson (1982), Bentley *et al.* (1983), Burkhart *et al.* (1984) and Dr M. Ashburner (*personal communication*) have identified the *cMdh*, *Adh*, *Idh*, *Est-C*, and *Me* nulls as low activity variants (Table 8.2). Calculations based on these corrections reduce the average null allele frequency found in the London and Raleigh populations from 0.24% to 0.11%.

An important point concerning the discrepancy between the Tasmanian and Raleigh/London frequencies is whether the Tasmanian populations are unique in some way. More specifically: (1) is there any evidence that the rate of mutation and/or the effect of selection at the *Adh* locus in the Tasmanian populations are any different to other natural populations

of *D. melanogaster*? and (2) do other loci encoding enzymes also show a high frequency of mutant alleles? Certainly, on the null frequency data alone, the Tasmanian populations differ from the London and Raleigh populations, and also from other Australian and New Zealand populations. For example, *Adh* null alleles were detected in collections from Rutherglen and Coffs Harbour, however, the frequency of nulls found at these locations, although high in comparison to the overseas reports, tended to be lower than that found in Tasmania during the same year. As well, collections of comparable sample sizes from Cardwell in Queensland and from Nelson, a New Zealand city of the same latitude as Tamar, did not contain *Adh* null alleles.

The only other enzyme locus which we have information for is *Gpdh*; unlike *Adh*, the level of electrophoretic and activity variation at this locus was consistent with the data of Langley *et al.* (1981) and Burkhart *et al.* (1984). Low activity *Gpdh* variants were found in the Tasmanian (1984) and the London/Raleigh samples at comparable frequencies (0.5% and 0.58% respectively). In addition, similar low activity *Gpdh* variants have been found in other Australian Queensland populations, including Cardwell, although none were detected in the sample from New Zealand (Dr J.B. Gibson, *personal communication*). Consequently, it is possible that the *Adh* locus is subject to some unique mutator activity, but more information is required about the null allele frequencies at other enzyme loci, both within Tasmania and in other Australian populations.

There is no evidence suggesting that the mutation rates in these four Tasmanian populations are any higher than in other Australian populations. On the contrary, little or no male recombination or P-element activity has been found in the Tasmanian populations in

comparison to Coffs Harbour where both do occur (Dr I. Boussy and Dr J.B. Gibson, *personal communication*). In addition, the frequency of second chromosome recessive lethals does not differ significantly between the Tasmanian and Coffs Harbour populations (Dr J.B. Gibson, *personal communication*). It is impossible to rule out high mutation rates due to mutator activity caused by other unknown chromosomal mobile elements, but the levels of mutation required to account for the observed frequencies would have to be very high; the average frequency of *Adh* nulls in the 1983 study suggested a mutation rate of 10^{-3}.

It is possible that, at some time in their past history, the Tasmanian populations have had elevated mutation rates. Mason, Valencia, Woodruff, and Zimmering (1985) found spontaneous sex-linked recessive mutation frequencies changed over time in laboratory cultures of *D. melanogaster*, apparently the result of genetic drift and seasonal variation. Rapid changes in mutation rates have also been reported in natural populations of *D. melanogaster*. Mukai, Baba, Akiyama, Uowaki, Kusakabe, and Tajima (1985) observed that the frequency of lethal-carrying second chromosomes in a Japanese population doubled within three years (16% to 32%), but within 10 years, this frequency had nearly returned to the original value (18%). This marked fluctuation in mutation rate was accounted for by the invasion of the presumed M-cytotype population by a P-type mobile element. The resultant hybrid dysgenesis increased the frequency of observable mutations which later declined as all the individuals in the population became contaminated by this factor. The exact nature of the element(s) is not yet known, however, several diagnostic properties of the P-M and I-R dysgenic models were not observed and this lead Mukai *et al.* (1985) to suggest that another chromosome element or system may be involved. It is

possible that the relatively high frequency of *Adh* nulls in the Tasmanian populations is a relic of a similar invasion event occurring in the past.

To whatever extent this has occurred, the persistence of null alleles in the populations requires an explanation as does any hypothesis involving the spread of the alleles from a single mutation event. If the separately extracted null alleles are copies of a single mutant, then this mutant is present at polymorphic frequencies. This together with its present day distribution implies a selective advantage and it is possible that the mutant is maintained via a heterotic effect. For this reason it is important to know if the same *Adh* null is present in other Australian populations, particularly at the All Saints winery where one null was detected, but not isolated. It is possible that the physiological role of the *D. melanogaster* ADH system may be such that, in these Tasmanian populations, there is little or no selective disadvantage in possessing a null *Adh* allele.

There are a number of observations relevant to this discussion. For example, the threshold value at which selection acts on ADH activity in natural populations may be lower than generally assumed. Gibson and Oakeshott (1982) suggest that both in the tolerance and utilisation of ethanol there may be physiological thresholds above which the level of ADH activity is unimportant and this level may be lower than that found in Adh^S homozygotes. *D. simulans* has *in vitro* levels of ADH activity, on several alcohol substrates, less than half that found in Adh^S homozygotes of *D. melanogaster* (Daggard, 1981). This species also has a much lower ethanol tolerance than *D. melanogaster* and is rarely found at winery sites containing relatively high concentrations of ethanol (McKenzie and Parsons, 1972; Gibson *et al.*, 1981). For that reason, it

could be argued that the threshold level for selection of ADH activity in similar alcohol-containing environments approximates the activity found in *D. simulans*. Heterozygotes of the Tasmanian nulls with a standard Adh^S allele were found to have activity levels about half that of a normal Adh^S homozygote and consequently higher than the *D. simulans* level.

Furthermore, ethanol tolerance experiments indicate that null/active heterozygotes have comparable mortalities to the active allele homozygotes. This is in agreement with the findings of Middleton and Kacser (1983) who found no difference in the survivorship of Adh^F/Adh^F, Adh^S/Adh^S, Adh^F/Adh^n, and Adh^S/Adh^n genotypes on media supplemented with ethanol at a concentration of 15%. In addition, these authors found no evidence for an *in vivo* basis for selection between these genotypes, as the metabolic flux of ADH (as measured by the conversion of ethanol to CO_2 and lipids) was the same for all genotypes despite the large range of *in vitro* ADH activities. It appears doubtful that the *in vitro* ADH activity differences are likely to contribute to fitness differences, at least in terms of ethanol metabolism.

This is consistent with the calculated selective disadvantage of null/active allele heterozygotes. Langley *et al.*(1981) calculated the selective disadvantage of null heterozygotes in their populations to be 0.0015. They used the equation $q = u/hs$ where q is the null allele frequency, u is the mutation rate to null alleles (from Voelker *et al.*, 1980b; 3.86×10^{-6}), h is a measure of dominance, and s is the reduction in fitness of the null homozygote. The average frequency of *Adh* nulls observed in the 1983 samples gave a figure of 0.0003. Although the hs term cannot be further resolved it does appear as if these nulls have little deleterious effect in heterozygotes.

Of course any arguments concerning selection at the *Adh* locus rest on the assumption that the effect of ADH variation on fitness is mediated via its catalytic activity in oxidising ethanol (Middleton and Kacser, 1983). Middleton and Kacser (1983) note two points relevant to this assumption. First, ADH has a broad specificity and its action on other alcohols may be more important. Second, the structure of the *Drosophila* enzyme is so different from other ADHs that it most probably had an independent origin, perhaps by the modification of another enzyme. If so, the primary function of the original enzyme rather than the ADH's dehydrogenase activity may be the more important component of fitness.

In any case, as pointed out by Gibson and Oakeshott (1982), there are a number of behavioural and physiological strategies that might be used to adapt to ethanol in the environment and which would not depend on ADH activity. At high ethanol concentrations oviposition-site preference related to ADH activity has been observed in *D. melanogaster* with *Adh* null flies laying eggs on mediums containing less alcohol (Hougouto, Lietaert, Libion-Mannaert, Feytmans, and Elens, 1982). However Gelfand and McDonald (1980) found that *Adh* null larvae do not show any avoidance behaviour to higher ethanol concentrations and it remains possible that another enzyme system or a shunt pathway is fulfilling the metabolic function of ADH in null flies. Such a situation exists in *Gpdh* null mutants; homozygotes lack the ability to fly because of disruption to the energy-producing glycerophosphate cycle of the insect flight muscle (O'Brien and MacIntyre, 1972; O'Brien and Shimada, 1974). However, after a year of culture one null homozygote strain regained the ability to fly despite the continued absence of measurable GPDH activity. Although the enzymatic activities related to

the energy-producing function of the glycerophosphate cycle have been examined, the nature of the alternative shuttle(s) has not yet been elucidated (O'Brien and Shimada, 1974; O'Brien and MacIntyre, 1978). Of course, the existence of an alternative shuttle may reflect the adaptive significance of a particular biochemical system; the consequences for a *Drosophilid* of not being able to fly are likely to be more severe than an inability to detoxify environmental ethanol.

A further factor which needs to be taken into account is that the Tasmanian populations suffer severe bottlenecks during the winter months of July and August. Temperatures during this period would certainly prevent breeding but probably some larvae or adults survive until spring. How these populations are recolonised each spring is not known, nor is the proportion of the original pre-winter population which survive to contribute. In addition, the seasonal character of the breeding resource and the opportunistic nature of *D. melanogaster* lead to some uncertainty about a population's identity. Whether it is better to regard collections between years as from the same or from different populations is open to question. What is surprising is the consistency of Adh^F and Adh^S allele frequencies between years and the continued persistence of null alleles in the populations examined.

The *Adh* null alleles were first detected in 1983 and they were still found to be present in collections from Tamar (0.9%) and Huonville II (3.1%) made in May 1986 (Dr J.B. Gibson, *personal communication*). The recent history of the Tamar Valley population is interesting and relevant to the interpretation of null allele frequency differences between years. In 1983 and 1984 samples of *D. melanogaster* were taken from populations breeding on grape pressings dumped at the rear of the main winery building. *Adh* null allele frequencies in these two years

were 0.7% and 0.5% respectively. In 1985, the grape pressings were dumped at a new site some 500m from the winery. No *Adh* null alleles were detected in samples from the population at this site, but in 1986 they were detected at a frequency of 0.9%. This emphasises the effect of sampling on population frequency estimates when dealing with a comparatively rare variant.

Taken together, these observations allow some tentative conclusions to be made about the occurrence and maintenance of the *Adh* nulls in the Australian *D. melanogaster* populations. Once arisen, by whatever means and in whatever population or populations, these alleles could be maintained as heterozygotes with little or no selective disadvantage to the carrier. As such, genetic drift is liable to play an important role in determining their frequencies, particularly at times following winter bottlenecks, when population sizes are low. The number of generations between bottlenecks may not be sufficient to allow selection to significantly alter null frequencies in the light of low heterozygous fitness differences. Selection against null alleles is likely to be only important in the case of null homozygotes and perhaps when environmental alcohol concentrations are high. Nevertheless, small fitness differences between null heterozygotes and active allele homozygotes, taken together with the mode of clinal selection associated with rainfall, may act to maintain consistent Adh^F and Adh^S allele frequencies and effectively limit the frequency of null alleles.

Table 8.1. Biochemical and molecular properties of the *Adh* null alleles isolated from the Tasmanian *D. melanogaster* populations (+ = yes; - = no).

Year sampled	Population	Null allele	Survival to pentenol vapour	ADH activity	Heterodimer formation	ADH protein	*Adh* gene region intact	Wild-type *Adh* mRNA
1983	Cygnet	AC14	+	-	-	-	+	-
1983	Cygnet	AC22	+	-	-	-	+	-
1983	Cygnet	AC41*	N.T.	-	-	N.T.	N.T.	N.T.
1983	Cygnet	AC53	+	-	-	-	+	-
1983	Cygnet	AC60*	N.T.	-	-	N.T.	N.T.	N.T.
1983	Cygnet	AC62*	N.T.	-	-	N.T.	N.T.	N.T.
1983	Cygnet	AC80	+	-	-	-	+	-
1983	Pipers Brook	AP81	+	-	-	-	+	-
1983	Tamar	AT33	+	-	-	-	+	-
1984	Cygnet	AC95	+	-	-	-	+	-
1984	Huonville II	AH36	+	-	-	-	+	-
1984	Huonville II	AH41†	+	-	-	N.T.	+	-
1984	Huonville II	AH108†	+	-	-	N.T.	+	-
1984	Tamar	AT240	+	-	-	-	+	-
1984	Tamar	AT265	+	-	-	-	+	-
1984	Tamar	AT340	+	-	-	-	+	-
1985	Huonville II	AH9	+	-	-	N.T.	+	-
1985	Huonville II	AH26	+	-	-	N.T.	+	-
1985	Huonville II	AH52†	+	-	-	N.T.	+	-
1985	Huonville II	AH70	+	-	-	N.T.	+	-
1985	Huonville II	AH77*	N.T.	N.T.	-	N.T.	+	-
1985	Huonville II	AH98	+	-	-	-	+	-
1985	Huonville II	AH144	+	-	-	N.T.	+	-

N.T. - not tested; *homozygous lethal; †homozygotes sterile

Table 8.2. Data from Langley *et al.* (1981) on null allele frequencies in London and Raleigh populations of *D. melanogaster* corrected for null alleles identified as low activity variants.

Autosomal locus	Number of null alleles	Number of alleles scored	Null allele frequency %	Number of null alleles found to have activity	Corrected null allele frequency %
Got-2	3	1139	0.26	0 (1)	0.26
Pgk	0	1131	0.00	-	-
Gpdh	10	1200	0.83	10 (1, 2)	0.00
cMdh	2	1223	0.16	2 (1)	0.00
Adh	1	1170	0.09	1 (3)	0.00
Pep-A	5	1147	0.44	0 (1)	0.44
Pgi	1	1152	0.09	0 (1)	0.09
Hex-C	2	1165	0.17	0 (1)	0.17
Idh	3	1366	0.22	1 (1, 5)	0.15
Est-6	0	1224	0.00	-	-
Pgm	1	1344	0.07	0 (1)	0.07
Est-C	6	1166	0.51	2 (1)	0.34
Odh	1	1173	0.09	0 (1)	0.09
Me	7	1147	0.61	3 (1, 4)	0.35
Xdh	0	976	0.00	-	-
Ao	13	1152	1.13	13 (1)	0.00
mMdh	0	1173	0.00	-	-
Ald	0	1350	0.00	-	-
Acph-1	3	1217	0.25	0 (1)	0.25
Tpi	0	1063	0.00	-	-
	58	23678	0.24	32	0.11

Numbers in brackets refer to references as follows:

(1) Burkhart *et al.*, 1984; (2) Dr J.B. Gibson, *personal communication*;

(3) Dr M. Ashburner, *personal communication*; (4) Williamson, 1982;

(5) Bentley *et al.*, 1983.

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